STUDIES ON WHITE SPOT SYNDROME (WSS) IN PENAEID PRAWNS FROM CULTURE PONDS AT COCHIN, INDIA

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DECLARATION

I here by declare that this thesis entitled **"Studies on white spot syndrome (WSS) in penaeid prawns from culture ponds at Cochin, India"** has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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CERTIFICATE

Certified that the thesis entitled "**Studies on white spot syndrome (WSS) in penaeid prawns from culture ponds at Cochin, India**" is a bonafide record of the work carried out by **Ms. Jasmin .K. Jose** under my guidance and supervision and that no part thereof has been presented for the award of any other degree, diploma or any other similar title.

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(Jasmin .K. Jose)

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

The aquaculture of penaeid shrimp has grown from its experimental beginnings roughly about three decades ago into a major industry which, on a world wide basis, provides employment to hundreds of thousands of both skilled and unskilled labourers, billions of U.S. dollars in revenue and a high quality food product (Lightner and Redman, 1998). Factors, such as, increasing demand and market value, introduction of new technologies in the prawn seed and feed production and other modern culture techniques contribute to this rapid development in the prawn culture industry, which in turn has been accompanied by the appearance of diseases of both infectious and non infectious aetiologies. Though the usage of the terms, prawns and shrimps has been much confusing, in the present study both the terms are used analogously to refer to marine/brackishwater forms only. The occurrence and relative importance of many of the penaeid diseases coincide with the type of culture system, i.e., extensive, semi intensive and intensive (based on the area of culture, stocking density, feeding and management) practised. The latter two are greatly employed on a commercial scale and hence have more prone to the unhealthy conditions and thereby to various diseases. Many of the important penaeid diseases are caused by organisms, that are part of the normal microflora and fauna of penaeids. These organisms are opportunistic pathogens, that cause diseases only under conditions that favour them over the host (Lightner, 1985, 1993).

The important infectious diseases of economic importance to the cultured prawn are those with viral, rickettsial, bacterial, fungal, protistan and metazoan aetiologies (Lightner, 1988, 1993; Brock and Lightner, 1990; Fulks

and Main, 1992; Johnson, 1995). A number of non infectious diseases are also of importance to the industry, which include diseases due to environmental stress, nutritional imbalances, toxicants and genetic factors (Lightner, 1988, 1996; Brock and Lightner, 1990; Brock, 1992; Johnson, 1995). Among all these, viruses are the most important disease causing agents in the penaeid prawns (Lightner, 1985).

The occurrence and intensity of viral infection vary with the prawn species, genetic strain and their life stages (Lightner and Redman, 1998). As many as eleven virus diseases of cultured penaeid prawn are recognised and described (Lightner, 1993). These viruses are: *Baculovirus penaei* or BP (Couch, 1974a, 1974b), *Penaeus monodon*-type baculovirus or MBV (Lightner and Redman, 1981), which includes Plebejus baculovirus or PBV (Lester et al., 1987), baculoviral midgut gland necrosis virus or BMN (Sano *et al.*, 1981), type C baculovirus of *Penaeus monodon* or TCBV (Brock and Lightner, 1990), haemocyte-infecting baculovirus of *P. monodon* and *P. esculentus* or HB (Owens, 1993), infectious hypodermal and hematopoietic necrosis virus or IHHNV (Lightner *et al.*, 1983a; Bonami *et al.*, 1990), hepatopancreatic parvo-like virus or HPV (Lightner and Redman, 1984; Lightner *et al.*, 1985), two forms of reo-like viruses, REO-3 and REO-4 (Tsing and Bonami, 1984) and lymphoid organ vacuolisation virus or LOVV (Bonami *et al.*, 1992).

In addition to these, several new diseases of viral aetiology have been reported in recent years. They are white spot group of viruses (Lightner *et al.*, 1998), yellow head virus or YHV (Limsuwan, 1991; Boonyaratpalin *et al.*, 1993; Chantanachookin *et al.*, 1993; Wongteerasupaya *et al.*, 1995b) and Taura syndrome virus or TSV. Including the viruses in penaeids, more than thirty virus diseases are known to occur in crustacea (Lightner, 1993).

In prawn aquaculture, the white spot syndrome baculovirus (WSBV), which has been found in many prawn species, crabs and lobsters has had a significant negative impact on the entire prawn farming industry, not just in Asia, but globally, by causing substantial production and economic losses (Takahashi *et al.*, 1994; Chou *et al.*, 1995; Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995a; Lightner, 1996; Lo *et al.*, 1996a, 1996b, 1997; Flegel, 1997). The rapid onset, wide host range and marked lethality of this disease have made it one of the most dreadful disease problems in the culture ponds now a days (Chou *et al.*, 1995, 1998; Chang *et al.*, 1996), which demands more attention and a much more deeper investigation in this field.

Penaeus monodon and *P. indicus* are the two major species cultured in India, the former accounting for more than 80% of the farming area (Venkatesan, 1996). Mass mortalities of cultured *P. monodon*, due to infection with monodon baculovirus (MBV) had been reported from different parts of India (Ramasamy *et al.*, 1995; Vijayan *et al.*, 1995; Sundararaj *et al.*, 1996; Murali Manohar *et al.*, 1996; Karunasagar *et al.*, 1998).

The basic and the most important fact lying behind any culture practice is that disease is an end result of a complex interaction of the prawn, its environment and the disease causing agent (pathogen) itself. As Snieszko (1973), pointed out, aquatic species ".... Are not in vacuum- we have them in their environments....". The host can vary in its species, strain, age, life stage, nutritional status etc.; the pathogen can vary in virulence, concentration, ability to circumvent the host's defences, etc. and the environment can vary from ideal (as far as the host is concerned) to one that favours the disease causing agent more (Lightner *et al.*, 1998) and thus, there will be a sudden or gradual occurrence of a disease.

In order to develop proper control measures for the penaeid disease, an early and accurate diagnosis is a must. The commonly used diagnostic methods for various pathogens include the traditional methods of morphological pathology (direct light microscopy, histopathology, electron microscopy), enhancement and bioassay methods, traditional microbiology and the application of serological methods. The need for rapid, sensitive diagnostic methods led to the application of modern biotechnology to penaeid prawn diseases (Lightner *et al.*, 1998), which include development of modern diagnostic genome probes.

Although, white spot syndrome (WSS) was reported in India in early 1993, it was only in 1994, that adequate attention was given to it as a consequence of its increased severity and large-scale mortality of prawns. The loss had been estimated to be about 10,000-12,000 tons, valued at Rs. 250-300 billion (Alagarswami, 1995). In 1995, the causative organism of the epizootic, occurred in India was identified for the first time as SEMBV (Anon., 1995). Thereafter, four reports were published on this viral epizootic from India (Murali Manohar *et al.*, 1996; Karunasagar *et al.*, 1997; Rajendran *et al.*, 1998, 1999). The susceptibility of four species of marine prawns to SEMBV (*Penaeus monodon, P. indicus, P. vannamei, Metapenaeus dobsoni*) had been reported. Susceptibility of some of the freshwater prawns, crabs and lobsters to WSSV was also investigated by Rajendran *et al.* (1999). A detailed work on WSS, including the ultrastructural aspects had been done in *P. monodon* and *P. vannamei*, because of their importance from the culture and economic point of view.

While the knowledge of viral diseases of crustaceans in general and of penaeid prawns in particular is fairly developed and progressive in the advanced countries, the information on the subject, especially WSS from India is very limited. However, the topic assumes utmost importance, as many of the

cultured and economically important crustaceans (both marine and fresh water prawns, crabs and lobsters) are affected by the epizootic, WSS. It is the need of the hour to study the various aspects of WSS and to devise the necessary prophylactic and control measures to prevent the occurrence of this epizootic. We can not afford to sacrifice the entire cultured stock, valuable labour and time of our farmers. The present work is carried out with the following objectives of studying:

- The role of various environmental factors in inducing and enhancing the occurrence of white spot syndrome (WSS) epizootic in the prawn culture ponds.
- Penaeid prawns affected with WSS, especially, *Penaeus indicus*, the size ranges affected mostly and the symptoms of the epizootic.
- The histopathological changes in the various target organs and tissues of the affected prawns.
- Study of the pathogen at the ultrastructural level and the related changes in the host cells.

An earnest attempt was made to fulfil the above mentioned objectives. The thesis contains three chapters. In the first chapter, an attempt was made to throw light on the effects, if any, of the various important environmental factors on the occurrence of the disease. Care was also taken to find out the size range of *Penaeus indicus* H. Milne Edwards and other species collected from the culture ponds, such as *P. monodon* Fabricius and *Metapenaeus dobsoni* (Miers) and the symptoms of the epizootic. The second chapter presents the histopathological changes in the target organs namely, subcuticular epidermis, gills, foregut epithelium, connective tissues, hindgut, heart and compound eyes. The ultrastructural alterations in the affected cells of the target organs are presented in the third chapter.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Prawn aquaculture has the potential to become a long term sustainable industry throughout the world. But its rapid growth has been accompanied by an increased awareness of the negative impact of diseases on the industry (Lightner, 1993). Valuable studies on penaeid prawn diseases had been contributed by Sindermann (1970, 1974, 1979, 1990), Overstreet (1973, 1982), Barkate *et al.* (1974), Johnson (1974, 1975, 1978, 1983, 1990), Lightner *et al.* (1975, 1989, 1992b), Delves-Broughton and Poupard (1976), Liao *et al.* (1977, 1987a, 1987b, 1992), Lightner (1977, 1983, 1985, 1988, 1993, 1996), Nurdjana *et al.* (1977), Perez Alvidrez (1977), Couch (1978), Soni (1986), Brock and Lightner (1990), Colorni (1990), Liu (1990), Owens and Hall-Mendelin (1990), Brock (1991), Chen (1992), Flegel *et al.* (1992), Shariff and Subasinghe (1992), Bashirullah and Aguada (1993) and Karunasagar *et al.* (1998).

Among the various infectious diseases of penaeid prawns, viral diseases are most important, due to the rapid spread, mass destruction of the stock and lack of proper remedial measures against the causative agents. The recently reported devastating epizootics due to various virus pathogens of penaeid shrimp have caused significant and sometimes catastrophic economic losses in commercial culture ponds (Lightner, 1988, 1992; Brock, 1991; Boonyaratpalin *et al.*, 1993; Takahashi *et al.* 1994; Stern, 1995; Fraser and Owens, 1996; Flegel, 1997). Studies conducted by Lightner (1983, 1993), Johnson (1984), Lightner and Redman (1991, 1992, 1998), Lightner *et al.* (1992c), Momoyama (1992), Flegel (1997), Lotz (1997), Nakajima (1997), Owens (1997), Spann and Lester (1997), Munday and Owens (1998), Sohn and Park (1998) Takahashi *et al.* (1998) and Wang and Dai (1999) gave general information on the viral diseases of penaeid

prawns. Until the late 1980s, only six viral diseases were known in cultured penaeid shrimp (Lightner, 1990; Shariff and Subasinghe, 1992). Since then, the number of viral species affecting shrimps have more than doubled and today, nearly twenty distinct viruses or groups of viruses, representing seven virus families are known to infect the penaeid prawns (Lightner and Redman, 1998). In several other conditions, viral involvement are strongly suspected. Viruses belonging to the monodon baculovirus (MBV), baculoviral midgut gland necrosis virus (BMNV), hepatopancreatic parvo-like virus (HPV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV) and yellow head virus (YHV) groups have been important pathogens of cultured shrimps in Asia and Indo-Pacific regions, while, IHHNV, *Baculovirus penaei* (BP) and taura syndrome virus (TSV) have been the principal viruses of concern in the Americas (Lightner *et al.*, 1998). The major viral diseases prevalent in our prawn culture farms are reviewed here.

IHHNV was first recognised in 1981 in cultured *Penaeus stylirostris* from Hawaii and was reported to cause catastrophic epizootic in semi intensively and intensively cultured juveniles of that species, especially in larvae weighing between 50 mg and 2.0 g approximately (Lightner *et al.*, 1983a, 1983b; Bell and Lightner, 1987; Brock and Lightner, 1990; Lightner, 1996). IHHNV infection was recorded in *P. vannamei*, *P. monodon*, *P semisulcatus*, *P. japonicus* and in a hybrid penaeid prawn (*P. esculentus* X *P. monodon*) of South east Asian countries, Australia and Americas (Lightner, 1985, 1988, 1993, 1996; Owens *et al.* 1992). As *P. setiferus*, *P. duorarum*, *P. aztecus* and *P. californiensis* have been infected experimentally (Lightner, 1993), they are prone to natural IHHNV infections. IHHNV has been identified as an etiological factor in "runt-deformity syndrome" (RDS) in cultured *P. vannamei* (Kalagayan *et al.*, 1991; Browdy *et al.*, 1993; Castille *et al.*, 1993).

The gross pathology, histopathology and cytopathology of IHHNV in penaeids had been studied by Lightner (1983, 1985, 1988, 1993, 1996) and Lightner *et al.* (1983a). It is the smallest of the known penaeid shrimp viruses (Bonami *et al.*, 1990; Adams and Bonami, 1991; Bonami and Lightner, 1991). Based on its molecular characteristics, IHHNV has been classified as a member of the family, Parvoviridae (Bonami *et al.*, 1990).

Penaeid shrimp surviving IHHNV infections became carriers of the virus for life and there was a chance for vertical transmission by infected broodstock (Lightner *et al.*, 1983b). Serological tests using monoclonal and polyclonal antibodies (Bonami *et al.*, 1990) and non radioactive DIG-labelled gene probes to IHHNV and PCR methods for the detection of virus had been developed (Lightner *et al.*, 1992a; Mari *et al.*, 1993; Lightner, 1996). Jimenez *et al.* (1999) related the occurrence of IHHNV epizootic in *Litopenaeus vannamei* (Boone) and *L. stylirostris* (Stimpson) during 1990s to the oceanographic and climatological conditions caused by *El Nino*. From India, there was only a single report on the occurrence of IHHNV in cultured *Penaeus monodon* in Tamil Nadu (Sheela *et al.*, 1998). The histopathological observations made were identical to the earlier reports by Lightner (1993).

Hepatopancreatic parvo-like virus (HPV) was first identified in wild banana shrimp, *Penaeus merguiensis* and later in *P. esculentus* from Australia (Paynter *et al.*, 1985; Roubal *et al.*, 1989). It has a wide geographic distribution in the Indo-Pacific region and is observed in a number of cultured and wild penaeid species, especially in *P. merguiensis*, *P. indicus*, *P. chinensis* (= *P. orientalis*), *P. monodon*, *P. semisulcatus* and *P. penicillatus* (Chong and Loh, 1984), (Lightner and Redman, 1985, 1991; Lightner *et al.*, 1985, 1987; Lightner, 1988; Owens,

1997). It was also reported in *Macrobrachium rosenbergii* from Malaysia (Anderson, 1988) and in *P. japonicus* postlarvae from a hatchery in Queensland (Spann *et al.*, 1997b). Accumulative mortality rates in *P. merguiensis* and *P. semisulcatus* reached as high as 50% to 100% respectively, within 4 to 8 weeks of the disease onset, especially in juvenile stages (Lightner, 1988).

Diagnosis of Hepatopancreatic parvo-like virus (HPV) is mainly by the histologic demonstration of single, prominent, basophilic, Feulgen-positive, PAS-negative, fine granular, intranuclear inclusion bodies in the hypertrophied nuclei of the hepatopancreatic tubule epithelial cells (Spann *et al.*, 1997b). Consequent lateral displacement and compression of the host cell nucleolus and chromatin margination of the infected nuclei are also very good characteristics of HPV infection (Lightner, 1985, 1993). Transmission electron microscopic studies of these intranuclear inclusion bodies revealed dense areas of granular virogenic stroma and present in the stroma were aggregates of usually spherical (but sometimes five or six sided), virus particles of 22 to 24 nm in diameter. Paracrystalline arrays of HPV has not been reported so far (Lightner and Redman, 1985, 1991; Lightner, 1988; Bonami and Lightner, 1991). According to Flegel *et al.* (1999), stunted shrimp were more susceptible to HPV infection. Transmission might be both by vertical and horizontal pathways (Chong and Loh, 1984; Lightner, 1993). Mari *et al.* (1995) partially cloned the genome of HPV and gene probes were developed for disease diagnosis. Using DNA probe, Lightner *et al.* (1994) observed HPV in *P. chinensis* and *Macrobrachium rosenbergii*.

Lymphoid organ parvo-like virus (LOPV or LPV) was one of the recently discovered virus of penaeid shrimps by Owens *et al.* (1991) in *Penaeus monodon, P. merguiensis* and *P. esculentus* from Australia. Histopathology of the

affected shrimps showed mild nuclear hypertrophy, marginated chromatin and basophilic darkening of increased cytoplasm in multinucleate giant cells of hypertrophied lymphoid organ. The characteristic giant cells often formed discrete, fibrocyte-encapsulated spherical structures. Transmission electron microscopic studies conducted by Owens and co workers (1991) revealed paracrystalline arrays of viral particles of 18-20 nm diameter, associated with inclusion bodies in the nucleus of the transformed lymphoidal cells. Though, LPV was somewhat similar to IHHNV in tissue tropism and presence of rare Cowdry type A inclusion bodies (Owens and Hall-Mendelin, 1990; Owens *et al.*, 1991), it did not react with a gene probe for IHHNV (Owens, 1997).

Spawner-isolated mortality virus (SMV) was first detected in black tiger shrimp spawners during 1992-1994 period from Australia and transmission to native shrimp was achieved by oral and parental routes (Fraser and Owens, 1996). Icosahedral, non-enveloped, DNA viral particles, 20 nm in diameter were detected in the nuclei of midgut epithelial cells, which were similar to a parvovirus (Fraser and Owens, 1996). From mid 1994, this virus had been associated with substantial mortalities of 25 to 50% in 12 to 15 g black tiger shrimps as a part of the mid-crop mortality syndrome, MCMS (Munday and Owens, 1998; Owens *et al.*, 1998). Also, enveloped, filiform virions were seen in the cytoplasmic, basophilic inclusion bodies in the hematopoietic tissue and lymphoid stromal matrix of shrimps affected with MCMS, which were similar to yellow head rhabdo-like virus (YHV) (Owens, 1997). A parvo-like virus had been identified in *P. japonicus* post larvae in Queensland and it occurred in the epithelium of the digestive gland, which contained prominent, intranuclear inclusion bodies, 5 to 12 μm in diameter (Spann and Lester, 1997). The virus particles in these inclusions

were smaller (17 to 20 nm in diameter), than those of HPV (21 to 24 nm in diameter) (Munday and Owens, 1998).

Baculoviruses were the most commonly found viruses in crustaceans, there being at least 12 named types. They were often associated with diseases in both penaeid prawns and portunid crabs (Johnson, 1988; Johnson and Lightner, 1988). The pathogenic baculoviruses of penaeid prawns included, type A occlusion body forming viruses, *Baculovirus penaei* (BP) and monodon baculovirus (MBV), and type C non occluded baculoviruses such as baculoviral mid gut gland necrosis virus (BMNV), type C baculovirus (TCBV), Owen's penaeid haemocyte infecting rod-shaped virus (PHRV) and white spot syndrome virus (WSSV) (Lightner, 1993). Until 1993, all the known penaeid baculoviruses were enteric, infecting the hepatopancreatic and mid gut epithelial cells of their host (Johnson and Lightner, 1988; Brock and Lightner, 1990; Adams and Bonami, 1991). But recently reported baculoviruses, PHRV and WSSV-type affected haemocytes and tissues of ectodermal and mesodermal origin (Owens, 1993; Wongteerasupaya *et al.*, 1995a) respectively. According to Lightner *et al.* (1992a), among the fore gut infecting baculoviruses of penaeid shrimps, BP and MBV infected 15 and 8 species respectively. The cytopathology of BP, MBV and BMN was generally similar and often the affected hepatopancreatocyte nuclei had a peripherally displaced, compressed nucleolus and marginated chromatin (Lightner, 1985).

Baculovirus penaei (BP) has been reported to cause severe disease in the larval, postlarval and early juvenile stages of several penaeid species of the American continent (Couch *et al.*, 1975; Couch, 1978, 1989; Lightner, 1985; Lightner *et al.*, 1985, 1989; Johnson and Lightner, 1988; Overstreet *et al.*, 1988;

LeBlanc and Overstreet, 1990; Bueno *et al.*, 1990; Krol *et al.*, 1990; Bonami *et al.*, 1995; Lightner, 1996). The important species affected are, *Penaeus duorarum, P. aztecus, P. vannamei, P. stylirostris, P. penicillatus, P. schmitti* and *P. subtilis* (Couch, 1974a, b; Johnson and Lightner, 1988; Overstreet *et al.*, 1988; Bueno *et al.*, 1990; LeBlanc and Overstreet, 1990, 1991; Couch, 1991; LeBlanc *et al.*, 1991; Lightner and Redman, 1991, 1992). BP has not been reported outside of the Americas and Hawaii (Lightner and Redman, 1992; Overstreet, 1994). A morphologically and genetically unique strain of BP occurred in wild *P. marginatus* in Hawaii (Lightner *et al.*, 1985, 1994; Brock *et al.*, 1986).

PvSNPV or BP infections were diagnosed by the demonstration of prominent tetrahedral occlusion bodies (TOBs) or polyhedral inclusion bodies in unstained squash preparations of hepatopancreas, midgut or faeces (Overstreet *et al.*, 1988; Lightner, 1992, 1993; Bruce *et al.*, 1994). Bonami *et al.* (1995) purified PvSNPV (=BP type virus) and partial characterisation and cloning of the genome were attained. Morphometric comparisons of BP virions and nucleocapsids from different geographic regions suggested that three distinct strains of BP probably occurred (Lightner *et al.*, 1985; Bruce *et al.*, 1993; Bonami *et al.*, 1995). *In situ* hybridisation and PCR methods were found to be more sensitive for the detection of BP in infected tissues and faeces than the classical methods of direct microscopy, histopathology and serology with polyclonal antibodies (Lewis, 1986; Bruce *et al.*, 1991, 1993, 1994; Bonami *et al.*, 1992, 1995; Lightner *et al.*, 1992c, 1994; Lightner,1996; Wang *et al.*, 1996).

Faecal contamination of eggs, eating of faeces and cannibalism of infected shrimps were responsible for spread of BP infection among shrimps (Sano *et al.*, 1985; Johnson and Lightner, 1988; Momoyama, 1988; Overstreet *et*

al., 1988; Momoyama and Sano, 1989; LeBlanc and Overstreet, 1990, 1991; Lightner, 1992, 1996; Sano and Momoyama, 1992). Experimental direct transmission of BP virus from shrimp to shrimp showed a 24 h incubation period (Overstreet *et al.*, 1988), which is similar to the incubation periods of MBV and BMNV (Momoyama, 1988; Lightner, 1996).

MBV-type baculoviruses had a diverse host range and wide distribution on the Indo-Pacific coasts of Asia, Australia, Africa, southern Europe, Philippines, Indonesia, Thailand, Singapore and Taiwan (Lightner and Redman, 1981; Lightner, 1985, 1993; Nash *et al.*, 1988; Chen *et al.*, 1989a; Baticados *et al.*, 1990; Fegan *et al.*, 1991; Flegel *et al.*, 1992; Lightner *et al.*, 1992b; Owens, 1997). It was pathogenic to *Penaeus monodon, P. penicillatus, Metapenaeus ensis, P. merguiensis, P. semisulcatus, P. kerathurus P. vannamei,* Eastern king prawns, *P. esculentus* and *P. plebejus* (Lightner and Redman, 1981, 1992; Lightner *et al.*, 1983c, 1985, 1987, 1990; Lester *et al.*, 1987; Doubrovsky *et al.*, 1988; Johnson and Lightner, 1988; Lightner, 1988; Chen *et al.*, 1989a, 1989b),

Lightner *et al.* (1983c) reported that the hepatopancreatic tubule and duct epithelium of post larvae, juveniles and adults and the anterior mid gut epithelium of very young post larvae became the target organs for MBV. Several papers had been published on the virogenesis and pathogenesis of MBV (Lightner and Redman, 1981; Lightner *et al.*, 1983c; Chen *et al.*, 1989a, 1989b; Vogt, 1992). Lu *et al.* (1996) described the morphogenesis of a membranous labyrinth (ML) in the hapatopancreatic epithelial cells of *P. monodon* infected with MBV. Chang *et al.* (1992) purified the occlusion bodies of MBV and studied its biochemical characteristics.

Not all strains of MBV caused disease, as Natividad and Lightner (1992) observed that Tahitian MBV was not virulent to *Penaeus monodon*. Spann and Lester (1996) reported a new baculovirus from wild, *Metapenaeus bennettae* from Australia, which resembled MBV in its ultrastructure and histological appearance, but gave negative results with *in situ* hybridisation test using a DNA probe for MBV. They designated this new baculovirus as MbSNPV or bennettae baculovirus (BBV). Johnson and Lightner (1988) reported six baculovirus-related nuclear viruses, known to attack the hepatopancreatic epithelium of decapod crustaceans, including penaeid shrimps, brachyuran crabs and anomuran crabs. Vickers *et al.* (1993) developed an impression smear method for the rapid detection of MBV in Australian prawns. The primer sequences of MBV for PCR amplification had been published by Chang *et al.* (1993). Lu *et al.* (1995a) detected MBV in *P. monodon* by *in situ* hybridisation.

Stress and other infections enhanced baculovirus infection. The infections were tolerated when culture conditions were optimal (Couch *et al.*, 1975; Lightner *et al.*, 1983c, 1987; Lightner, 1985; Anderson *et al.*, 1987; Nash *et al.*, 1988; Chen *et al.*, 1989b; Halder *et al.*, 1989, Fegan *et al.*, 1991). MBV infected *P. monodon* displayed slower growth rates and exhibited generally pale yellow to reddish brown hepatopancreata, characterised by necrosis and degeneration of hepatopancreatic tubules (Baticados *et al.*, 1991).

In India, MBV had been reported from east and west coasts (Ramasamy *et al.*, 1995; Vijayan *et al.*, 1995; Sundararaj *et al.*, 1996; Murali Manohar *et al.*, 1996; Karunasagar *et al.*, 1998). The male and female broodstocks of *P. monodon* obtained from the Andaman sea of India exhibited 5.7% MBV infection (Fegan *et al.*, 1991). Transmission electron microscopic

studies conducted by Ramasamy *et al.* (1995) revealed rod-shaped, non-occluded or occluded and enveloped virions within a paracrystalline occlusion body.

Of the type C baculoviruses in prawns, extensive studies had been made on the various aspects of BMNV in *Penaeus japonicus*, including the morphology, ultrastructure, DNA analysis, methods of transmission, host ranges, diagnostic techniques and countermeasures (Sano *et al.*, 1981, 1985; Momoyama, 1983, 1988, 1989a, 1989b, 1989c, 1989d; Momoyama and Sano, 1988, 1989, 1996; Sano and Momoyama, 1992; Arimoto *et al.*, 1995). *P. monodon*, *P. chinensis* and *P. semisulcatus* were experimentally infected with BMNV by Momoyama and Sano (1996). BMNV was virulent to early life stages of the shrimp (Sano *et al.*, 1985; Momoyama and Sano, 1989). So far, this disease was confined to Japan alone (Lightner and Redman, 1991) and was characterised by a sudden onset and a high mortality rate in the larval population of *P. japonicus*.

Owens (1993) described the first penaeid haemocytic rod-shaped virus (PHRV) in hybrid prawns (*Penaeus esculentus* male crossed with *P. monodon* female), from Australia. Records of three haemocyte-infecting, rod-shaped viruses, similar to baculoviruses were restricted at present to the portunid crabs of the Atlantic Ocean (Johnson, 1988). Lightner and Redman (1993) described for the first time, the occurrence of a putative iridovirus in the penaeid shrimp, *Protrachypene precipua* Burkenroad from the sea water supply canal of a commercial shrimp farm in Ecuador.

Taura syndrome virus (TSV) is one of the most recently characterised penaeid shrimp viruses. It was first reported from cultured *Penaeus vannamei*, in farms located near the mouth of Taura River in Ecuador in 1992 (Jimenez, 1992) and later reports came from every major shrimp growing region in the Americas (Lightner, 1996; Brock *et al.*, 1997; Hasson *et al.*, 1997; Lightner *et al.*, 1997a,

1997b). Cumulative TSV-caused losses of cultured *P. vannamei* crops had been estimated at US $ 1.2 to 2 billion for the years, 1992 to 1996 (Lightner, 1995, 1996; Hasson, 1998). The susceptibility of different life stages of *P. vannamei* and *P. setiferus* to TSV infection was studied by Brock *et al.* (1995), Lightner *et al.* (1995), Lightner (1996) and Overstreet *et al.* (1997). Infectivity bioassays with the Asian penaeids, *P. monodon, P. japonicus* and *P. chinensis* suggested that *P. chinensis* juveniles were moderately susceptible to TSV, whereas, the other two were highly resistant (Brock *et al.*, 1997).

The current diagnostic and detection methods for TSV included, histopathology, *in situ* hybridisation, bioassay with susceptible juvenile *P. vannamei* and RT-PCR (Lightner and Redman, 1998; Nunan *et al.*, 1998b). Histopathology revealed acute lesions and multifocal areas of necrosis in five principal anatomical regions of infected *P. vannamei*, i.e. cuticular epithelium of gills, appendages, foregut, hindgut and general body cuticle (Lightner *et al.*, 1994, 1995). The lesion was characterised by the presence of several to numerous, variably sized, eosinophilic to basophilic cytoplasmic inclusion bodies (Brock *et al.*, 1995; Hasson *et al.*, 1995, 1997; Lightner *et al.*, 1995; Lightner, 1996). Studies by Hasson *et al.* (1999) provided a very good picture of the different stages of lesion development and disease cycle of TSV in *P. vannamei*. The aetiology of TS disease (toxic vs. a viral aetiology) had been a controversial issue, since the discovery of TSV in 1994 (Brock *et al.*, 1995; Hasson *et al.*, 1995). TSV had been classified with the Picornaviridae, based on its morphology, its cytoplasmic replication and other molecular characteristics (Hasson *et al.*, 1995; Lightner, 1996; Bonami *et al.*, 1997). TSV-specific genomic probes were developed by Mari *et al.* (1998) and Poulos *et al.* (1999) produced monoclonal (MAb) and polyclonal (PAb) antibodies against TSV. Transmission of the virus by cannibalism, passive transmission by insects and birds, and vertical

transmission were likely for TSV (Hasson *et al.*, 1995; Lightner, 1996; Garza *et al.*, 1997; Lightner *et al.*, 1997a)

REO-like viruses had been observed in *Penaeus japonicus* (Tsing and Bonami, 1984, 1987; Tsing *et al.*, 1985; Lightner, 1988), *P. monodon* (Anderson *et al.*, 1987; Nash *et al.*, 1988), *P. chinensis, P. vannamei* (Krol *et al.*, 1990) and *Palaemon elegens* (Vogt, 1994). REO-like infections in every reported case was found to be associated with mixed infections by other viruses (Nash *et al.*, 1988; Krol *et al.*, 1990), rickettsiae (Nash *et al.*, 1988; Vogt, 1994), or fungi (Tsing *et al.*, 1985; Tsing and Bonami, 1987; Lightner, 1988) and other disease syndromes (Lightner *et al.*, 1984). Recent works on the characterisation of reo-like viruses in penaeid shrimps from Asia and America revealed the existence of two, morphologically and structurally distinct types (Tsing and Bonami, 1987; Adams and Bonami, 1991; Lightner, 1993). Transmission electron microscopic studies were conducted by Brock and Lightner (1990), Krol *et al.* (1990) and Lightner *et al.* (1992a).

Owens (1997) had reported a gut and nerve syndrome (GNS) in the wild caught banana prawn, *Penaeus merguiensis* from Queensland, which had also been found to be associated with substantial losses of farmed *P. japonicus* in Australia (Munday and Owens, 1998). According to Lightner (1996), this syndrome had been related with a reovirus that caused mortalities in *P. japonicus* in Hawaii and France.

Severe lesions of the "Oka organ" or lymphoid organ, including extreme hyperplasia and metastasis were noted in *Penaeus monodon* from farms in Malaysia (Anderson *et al.*, 1987), Taiwan (Lightner *et al.*, 1987), Australia (Owens *et al.*, 1991), in *P. esculentus* (Paynter *et al.*, 1985), *P. vannamei, P. stylirostris* and *P. penicillatus* (Lightner *et al.*, 1987), and *P. chinensis* (Brock and

Lightner, 1990). Based on the morphological and biochemical characteristics, it had been classified as a togavirus and named as lymphoid organ vacuolovirus, LOVV (Bonami *et al.*, 1992).

Limsuwan (1991) first reported yellow head disease in pond-reared *Penaeus monodon* in the Central Thailand. Research on yellow head virus in Thailand had been reviewed by Flegel *et al.* (1995, 1997) along with current practices for diagnosis, prevention and control. In 1992, in Thailand, the pond harvest losses attributed to YHV were estimated to be 30 million US $ (Anon. 1993). Yellow head virus had wide distribution in cultured *P. monodon* and *P. japonicus* in the southeast Asian and Indo-Pacific countries (Flegel *et al.*, 1995, 1997; Lightner, 1996; Wang *et al.*, 1996; Flegel, 1997; Lightner *et al.*, 1997). Lu *et al.* (1994, 1995b) and Lightner *et al.* (1998) experimentally infected western hemisphere penaeid shrimps (*P. stylirostris, P. vannamei, P. aztecus, P. duorarum, P. setiferus*) with YHV and studied distribution of the virus in various tissues. The disease resulted in a cumulative mortality of 100% within 3 to 5 days, from the onset of the infection in black tiger prawns (Direkbusarakom *et al.*, 1998). Limsuwan (1991), Flegel *et al.* (1992, 1995), Boonyaratpalin *et al.* (1993), Chantanachookin *et al.* (1993), Kasornchandra *et al.* (1993), Brock and Main (1994) and Wang *et al.* (1996) worked on the histopathological changes induced by YHV on the ectodermal and mesenchymal tissues. Based on the transmission electron microscopic studies, and *in situ* hybridisation, Wongteerasupaya *et al.* (1995b) confirmed that the YHV of *P. monodon* is an RNA virus. Nadala *et al.* (1997) reaffirmed the classification of YHV as a rhabdovirus-like pathogen of penaeid shrimp. Wongteerasupaya *et al.* (1997) developed an RT-PCR amplification method to detect YHV of *P. monodon.* Direkbusarakom *et al.*

(1998) studied the virucidal effect of *Clinacanthus nutans* against YHV in *P. monodon.*

From India, the first report of yellow head disease was made by Shankar *et al.* (1994) in farm-reared *Penaeus monodon* at Andhra Pradesh, as a co-infection with white spot disease, resembling the co-infection of tiger shrimp with YHV and SEMBV in Thailand (Wongteerasupaya *et al.*, 1995a). Shankar *et al.* (1994) and Shankar and Mohan (1998) noticed the disease in sub-adults and adults of *P. monodon*, ranging in age from 45 to 60 days in grow-out ponds. Histopathology of cultured *P. monodon* Fabricius, showing gross signs of yellow head syndrome and white spot syndrome from the 1994 epizootic, was studied by Mohan *et al.* (1998). Mohan and Shankar (1999) described the occurrence of unusual giant cells in the heart of these infected prawns.

Lymphoid organ virus (LOV) was found in the broodstocks, juveniles and adults of *Penaeus monodon* in the grow-out ponds of Australia (Spann *et al.*, 1995; Spann and Lester, 1997). Lymphoid organ and gills were the tissues infected with this presumptive rhabdo-like-virus. Histopathological observations and ultrastructural studies were made by Spann *et al.* (1995).

Recently, Spann and Lester (1997) identified a gill-associated virus (GAV) in Queensland, where it had caused significant mortalities in the juvenile and adult cultured black tiger prawn and a high proportion of wild spawner. By studying histopathology and transmission electron microscopy of GAV, the above workers and Cowley *et al.* (1999) pointed out many similarities between GAV, YHV and LOV.

Lu *et al.* (1991) reported the isolation of a rhabdovirus (RPS) from *Penaeus stylirostris* and *P. vannamei.* Nadala *et al.* (1992) also observed the

histopathological changes and viral replication in the "Oka organ" (lymphoid organ) of subadult *P. stylirostris*, which were experimentally infected with rhabdovirus of penaeid shrimp (RPS). They also conducted infectivity and organ distribution studies of RPS. Lu *et al.* (1994) also conducted infectivity and organ distribution studies of RPS in the penaeid prawns.

Penaeid acute viremia (PAV), caused by non-occluded, bacilliform, enveloped, penaeid rod-shaped DNA virus (PRDV) was first observed in the spring of 1993, in cultured kuruma shrimp, *Penaeus japonicus* in Japan and it resulted in 60 to 80% of total losses (Inouye *et al.*, 1994, 1996; Takahashi *et al.*, 1994, 1996). Workers such as Inouye *et al.* (1994), Momoyama *et al.* (1994) and Nakano *et al.* (1994) opined that this was due to the introduction of PRDV infected seeds from China. Later it was reported in the seedling production of the greasyback shrimp (*Metapenaeus ensis*), *P. japonicus* from Japan (Momoyama *et al.*, 1997; Mushiake *et al.*, 1998; Satoh *et al.*, 1999) and in the cultured penaeid shrimps of Asia (Takahashi *et al.*, 1994, 1996; Lo *et al.*, 1996a). PRDV had a wide host range in decapod crustaceans, including shore crabs (*Helice tridens, Hemigrapsus sanguineus*), wild caught shrimp spawners and mud shrimp, *Upogebia major* (Maeda *et al.*, 1998a). Numerous virus particles were also observed in the hemolymph of moribund shrimps, causing viremia (Momoyama *et al.*, 1995) and hence, Inouye *et al.* (1996) proposed the name penaeid acute viremia (PAV) for the disease, caused by PRDV. Maeda *et al.* (1998a), identified the tissue tropism of PRDV in *P. japonicus*. According to Mushiake *et al.* (1999), the detection rate of PRDV was always higher in the receptaculum seminis of infected *P. japonicus* than in the ovary.

Diagnosis of PRDV was possible with dark field microscopy (Momoyama *et al.*, 1995) and PCR techniques (Kimura *et al.*, 1996; Takahashi *et*

al., 1996). Venegas *et al.* (1999) studied the pathogenecity of PRDV and demonstrated that the PRDV susceptibility of *P. japonicus* increased with the progress in developmental stages. The total number of PRDV-positive shrimp was significantly higher in kuruma spawners than in immature shrimps. Maeda *et al.* (1998a) and Takahashi *et al.* (1998), conducted infection trials and suggested that PRDV can be transmitted by both horizontal and vertical routes. Pathogenecity of PRDV to juveniles of six economically important crustacean species was examined by infection trials, conducted by Momoyama *et al.* (1999). PRDV from *P. japonicus* in Japan and SEMBV from *P. monodon* in Thailand, the two causative agents of white spot syndrome (WSS), were tested by Maeda *et al.* (1998b) for their sensitivities to chemicals, temperature, drying and singlet oxygen ($^1O_2$). Henning *et al.* (1998) examined the changes provoked by PAV in hemolymph parameters of peptidoglycan (PG)-fed kuruma shrimp.

Huang *et al.* (1995) described the histopathology of a shrimp explosive epidemic disease (SEEDS), caused by a hypodermal and hematopoietic necrosis baculo-like virus (HHNBV). During 1992-1993 period, it caused severe losses in Chinese shrimp culture. The pathological changes noted were, intranuclear vacuolation in the epithelial and connective tissues of the stomach and gill of the diseased shrimp with Trypan blue-Eosin Y (T-E) staining. The cell nuclei of seriously affected shrimp became inflated and eosinophilic (Cai *et al.*, 1995; Jie *et al.*, 1995).

White spot syndrome baculovirus, WSSV (PmNOB II-type) is a recently discovered group of crustacean viruses (Lightner, 1996; Nunan and Lightner, 1997), under which come the very closely related or more or less the same viruses, such as systemic ectodermal and mesodermal baculo-like virus, SEMBV (Wongteerasupaya *et al.*, 1995a), rod-shaped virus of *Penaeus japonicus*,

RV-PJ (Inouye *et al.*, 1994; Takahashi *et al.*, 1994), or penaeid rod-shaped DNA virus, PRDV (Inouye *et al.*, 1996), hypodermal and hematopoietic necrosis baculo-like virus, HHNBV (Huang *et al.*, 1994, 1995), which is an agent of shrimp explosive epidemic disease (SEEDS) and white spot baculovirus, WSBV (Chou *et al.*, 1995; Wang *et al.*, 1995; Durand *et al.*, 1996; Lightner, 1996). All these diseases were characterised by the appearance of white spots on the cuticle and nearly 100% mortality within 4 to 6 days, because of the high pathogenecity of the virus (Maeda *et al.*, 1998a). In 1994, outbreaks of a disease, that caused serious mortality of the black tiger shrimp, *Penaeus monodon* was reported from Thailand (Asian Shrimp Culture Council, 1994).

Wongteerasupaya *et al.* (1995a) accidentally discovered along with yellow head virus (YHV), a non-occluded, systemic baculovirus that occurred in the cells of ectodermal and mesodermal origin, in the black tiger prawn, *Penaeus monodon* from Thailand. This virus had a wide host range among *P. monodon, P. chinensis, P. merguiensis, P. indicus, P. vannamei* and *P. japonicus* (Asian Shrimp Culture Council, 1995; Wang *et al.*, 1995). Wongteerasupaya *et al.* (1995a) studied the molecular details of the purified DNA from the new virus and named it as SEMBV. It was included in the family, baculoviridae and subfamily Nudibaculovirinae as PmNOB II. A similar ultrastructure and nucleic acid content were subsequently described for WSV infections in *P. monodon* in Taiwan, where the causative virus was named, PmNOB III (Chou *et al.*, 1995; Wang *et al.*, 1995).

A specific DNA probe was developed by Peng *et al.* (1995) to detect SEMBV by dot blot hybridisation. As the infections caused by PRDV (RV-PJ) and SEMBV had the same clinical signs, target tissues and histopathological changes, except for the formation of inclusion bodies in SEMBV, these two types of viruses were closely related and both could be detected by PCR, using DNA

primers developed from PRDV (Takahashi *et al.*, 1996). Copepods and other crustaceans were known to be carriers of SEMBV (Yu and Wang, 1995).

In India, SEMBV was reported in *Penaeus monodon* from Tamil Nadu and Andhra Pradesh (Murali Manohar *et al.*, 1996; Sahul Hameed *et al.*, 1998; Mohan *et al.*, 1998; Shankar and Mohan, 1998). Sahul Hameed *et al.* (1998) experimentally infected *P. monodon* and *P. indicus*, using isolated and purified SEMBV and also devised an immunological method for rapid detection of the virus in the shrimp, without sacrificing the animal.

Similar virus diseases, closely related to PAV and SEMBV had been reported from several Asian countries and USA in different names (Baozhen *et al.*, 1995; Chou *et al.*, 1995; Huang *et al.*, 1995; Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995a; Lightner, 1996; Kasornchandra *et al.*, 1998). These syndrome diseases are generally known as white spot syndrome (WSS) and its agent(s) as white spot syndrome baculovirus (WSSV) (Lightner, 1996, 1999; Durand *et al.*, 1997). According to Inui (1996), PRDV and PAV are standardised names of fish diseases in Japan, while the term, 'WSSV' is widely used the world over. White spot syndrome (WSS) or red disease was first reported from cultured shrimp in Taiwan and Asia in 1992 (Chou *et al.*, 1995) and later spreaded throughout the east and southeast Asia, Indonesia, India and other shrimp growing countries (Inouye *et al.*, 1994, 1996; Momoyama *et al.*, 1994; Takahashi *et al.*, 1994; Anon., 1995; Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995a; Kasornchandra *et al.*, 1998). Prior to 1995, there was no report of WSSV and YHV from western hemisphere. Later, the occurrence of WSSV was detected in Texas and S. Carolina in *P. setiferus* (Rosenberry, 1996; Lightner *et al.*, 1997a; Nunan and Lightner, 1997; Nunan *et al.*, 1998a).

WSSV had been reported to infect many penaeid species and other arthropods (Lo *et al.*, 1996b; Maeda *et al.*, 1997; Momoyama *et al.*, 1997; Wang *et al.*, 1997b; Kasornchandra *et al.*, 1998). *Penaeus monodon, P. chinensis, P. indicus, P. penicillatus* and *P. japonicus* were the major species, naturally infected by WSSV in Asian countries (Chen, 1992; Kasornchandra and Boonyaratpalin, 1996). Additional hosts, which showed high susceptibilities to WSSV under natural or experimental conditions included, *Metapenaeus ensis, P. aztecus, P. duorarum, P. merguiensis, P. semisulcatus, P. stylirostris, P. vannamei, Trachypenaeus curvirostris* (almost all penaeid shrimps), *Exopalaemon orientalis, Macrobrachium rosenbergii* (carideans), *Procambarus clarkii* and *Orconectes punctimanus* (crayfish) (Cai *et al.*, 1995; Chang *et al.*, 1998; Lightner *et al.*, 1998; Wang *et al.*, 1998a, 1999). WSSV had also been detected in wild crabs (*Calappa lophos, C. philarigus, Portunus sanguinolentus, Charybdis sp., P. pelagicus, Helice tridens*), wild lobsters (*Panulirus sp.*), Palaemonidae, pest shrimp, copepoda, plankton, *Artemia* and pupae of an Ephydridae insect by PCR, *in situ* hybridisation or monoclonal antibody assays (Huang *et al.*, 1995; Lo *et al.*, 1996b; Chang *et al.*, 1998; Wang *et al.*, 1998a). Flegel (1996) and Lo *et al.* (1996a) had presented a list of susceptible species of WSSV.

The principal clinical sign of WSS was the presence of white spots of 0.3 to 3 mm size in the exoskeleton or cuticle of the diseased shrimp (Durand *et al.*, 1996, 1997; Wang *et al.*, 1997b; Lo and Kou, 1998). But in the diseased *Penaeus penicillatus* and *Macrobrachium rosenbergii*, usually white spots could be seen only after the carapace had been removed, while in portunid crabs, such as, *Scylla serrata*, the gross signs of WSS took the unusual form of clouded areas in the last two segments of the fifth pereiopod (Lo *et al.*, 1996a; Peng *et al.*, 1998a). Experimental studies conducted by Sahul Hameed *et al.* (2000) proved that *M. rosenbergii* is highly resistant to WSSV infection. Wang *et al.* (1997a) did

not observe any white spots in the carapace of infected *Metapenaeus ensis*. WSSV was highly tissue specific, infecting tissues of ectodermal (cuticular epidermis, gill tissue, nerve, antennal gland, foregut, hindgut, etystalk etc.) and mesodermal origin (striated muscle, heart, gonads, lymphoid organ, hematopoietic tissue, haemocytes, connective tissue etc.) and pleopods and pereiopods (Wongteerasupaya *et al.*, 1995a; Durand *et al.*, 1996; Lo *et al.*, 1997; Momoyama *et al.*, 1997; Wang *et al.*, 1997a; Lo and Kou, 1998). Chen *et al.* (2000) worked out the histopathology and found that tissue tropism in *Scylla serrata* crab larvae is similar to that found in shrimps.

Wang *et al.* (1997b) examined the morphology and composition of white spots on the inside surface of the carapace and opined that white spots are derived from the abnormalities of the cuticular epidermis. Wang *et al.* (1995) successfully purified WSBV from diseased black tiger shrimp. DNA diagnostic probes for WSBV had been developed in several laboratories (Chang *et al.*, 1996, 1998; Durand *et al.*, 1996; Wongteerasupaya *et al.*, 1996; Lo *et al.*, 1997; Kasornchandra *et al.*, 1998; Lightner and Redman, 1998; Hsu *et al.*, 1999; Otta *et al.*, 1999; Zhan *et al.*, 1999; Tang and Lightner, 2000; Van *et al.*, 2000) and the primers for detection by PCR technology had been published by Lo *et al.* (1996a). Huang and Song (1999) studied the possibility of maternal transmission of WSSV in *P. monodon*. An extensive study was made by Peng *et al.* (1998b) on the changes occurred during the transition from pre-patent white spot infection to patent infection in *Penaeus monodon*.

Lo *et al.* (1999) and Tapay *et al.* (1999) conducted a dot blot hybridisation and PCR analysis with clinical samples of WSSV from different geographical locations (China, India, Thailand, U.S., S. Carolina and Texas) and suggested that they were genetically related. An infectious virus disease causing

mass mortalities was reported in cultured *P. orientalis* from Korea, and the histological and transmission electron microscopic studies were conducted by Park *et al.* (1998) and according to them the Korean agent combined characters described for WSSV and PRDV.

According to Nunan *et al.* (1998a), transmission of exotic pathogens occurred through a variety of means, including migration with humans and animals, rapid transit by land, sea or air or through the shipment of infected frozen food particles. They detected WSSV and YHV in frozen commodity shrimps imported into United States from Asia and by using these, they could reproduce the disease in the indicator shrimp (*P. stylirostris*). Introduction of viruses to regions, where they previously did not occur has had catastrophic consequences to the local shrimp industry (Lightner *et al.*, 1983a, 1983b; Rosenberry, 1988). Experiments conducted by Chou *et al.* (1995, 1998) and Chang *et al.* (1996), Kanchanaphum *et al.* (1998) and Sahul Hameed *et al.* (2000) indicated that WSSV could be transmitted orally as well as via, water across shrimp species and other arthropods. Some of the wild decapods, which were used as feed for the broodstock and those seen along with the culture ponds and inlet canals, were also susceptible to WSBV infection. They acted as reservoir hosts to the virus and now a days, they could be easily detected by the diagnostic probes (Lo *et al.*, 1996b; Chang *et al.*, 1998; Supamattaya *et al.*, 1998; Wang *et al.*, 1998a).

The prevalence of WSBV had been shown to be high in wild brooders in Taiwan (Lo *et al.*, 1997; Lo and Kou, 1998). Although, there was no direct evidence of vertical transmission of WSBV, some infected spawners, in fact produced WSBV-positive larvae (Lo *et al.*, 1997). According to Lo and Kou (1998), transovum transmission of the virus was a very real possibility and the most effective preventive measure was to rinse or disinfect the nauplii. Screening

to produce specific pathogen-free (SPF) broodstock was now one important strategy for viral disease control in shrimp aquaculture (Wyban *et al.*, 1992). The effectiveness of the dietary incorporation of β-1,3-glucan from *Schizophyllum commune* in enhancing the resistance of post larval and juvenile *P. monodon* to WSSV was experimentally proved by Chang *et al.* (1999).

Flegel *et al.* (1996) and Wang *et al.* (1997b) examined the role of environmental parameters, such as, dissolved oxygen, pH, salinity, hardness that caused osmotic stress in culture ponds, and ammonia in enhancing the infectivity of WSSV in culture ponds. Based on the experiments conducted, Peng *et al.* (1998b) opined that, though, pre-patent stage was persisted for months, the transition to patent stage could occur within a few hours under stressful conditions. This indicated the quick multiplication and spread of WSSV in *P. monodon*, when triggered by a stressor. Although, Lightner (1996) reported that, so far, no significant resistance to WSS had been seen for any species of shrimps, there were instances when infected shrimp with white spots survived indefinitely, under non-stressful conditions (Chou *et al.*, 1995; Lo and Kou, 1998; Tsai *et al.*, 1999). Flegel (1997) also supported this by pointing out that in Thailand, many farmers obtained good to excellent harvests (4-7 tons/ha), in spite of finding a few specimens with gross signs of WSBV infection.

PCR technique is suitable for monitoring cultured shrimp during their grow-out period and also for examining broodstock for WSSV infection (Lo *et al.*, 1996a, 1996b, 1997) and this periodic checking in the cultured shrimp population is an important procedure in the control of the disease. According to Clifford (1999), the three most important elements to control WSSV in shrimp farms are: stocking WSSV-free postlarvae in the ponds, excluding the virus from the culture

system and reducing stress to the shrimp, which may trigger an outbreak of the disease.

Although, WSS occurred in India in early 1993, it was only in 1994, that adequate attention was paid to it as a consequence of its increased severity and large-scale mortality. During 1994-1995 period, white spot syndrome caused mass mortalities of cultured shrimps, *Penaeus monodon* and *P. indicus* along the east coast of India (Anon. 1995). The loss had been estimated to be about 10,000-12,000 tons valued at Rs. 250 to 300 billion (1US $ = Rs. 38.0) (Alagarswami, 1995). This disease had also spread to the West Coast, causing serious mortalities (Karunasagar *et al.*, 1997). When the disease broke out towards the end of 1994, a crop holiday was declared, but when the culture resumed, the disease continued to cause mortalities in the farms. Nair (2000) studied the history and present status of white spot disease in India.

Histopathological changes, indicative of WSBV infection were detected in wild stock of penaeid prawns and crabs by Sudha *et al.* (1996), Karunasagar *et al.* (1997, 1998) and Rajan *et al.* (2000). They also observed moderate to heavy septicaemia in moribund prawns. Adrish Sen *et al.* (1996) isolated and partially characterized the WSV in penaeids and an epidemiological investigation of white spot disease outbreak was carried out in shrimp farms along coastal Karnataka by Hegde *et al.* (1996). Rajendran *et al.* (1999) experimentally induced WSSV infection in shrimps, freshwater prawns, crabs and lobsters.

Nadala *et al.* (1998) characterised a non-occluded Chinese baculovirus (CBV) in *Penaeus japonicus*, measuring 322 to 378 nm in length and 130 to 159 nm in diameter. The purified nucleoprotein exhibited a unique striated structure as a result of stacking of ring-like structures and they studied them at the molecular level. Three separate isolates of white spot virus (WSV), purified from three

different penaeid species (*P. japonicus* from China, *P. monodon* from Indonesia and *P. setiferus* from U.S.) were compared biochemically and genomically by Nadala and Lo (1998).

From the above review of literature, it is very vivid that new viral epizootics are emerging year after year with the rapid intensification of the prawn culture industry world-wide. Early detection of such epizootic onsets is thus as critical as proper diagnosis of them. The present diagnostic procedures for the penaeid virus disease are largely dependent upon microscopic and histological demonstration of the particular cytopathology, that is unique to each disease. Better, more rapid and more sensitive diagnostic procedures, using tissue cultures, serologic methods and gene probes have been and are being developed. To date, however, none of these newer diagnostic techniques is inexpensive, simple to run or available in other than a few specialised laboratories. Electron microscopy is, so far, the most reliable method in diagnostic application.

Eventhough, WSD had been studied in detail in many penaeid prawns, very little attention had paid on the disease in *Penaeus indicus*. Many eminent workers in abroad have isolated, purified and gene probes had been developed for WSBV observed in *P. monodon, P. vannamei, P. stylirostris* etc. But, as a whole, information regarding the occurrence of WSD and the causative factors involved is very scanty from the prawn culture ponds of India. As, no treatments against viral infections are known and due to the present difficulties in diagnosing prawn as pathogen-free, the practice of transferring non-native species from distant geographic regions should be discouraged.

CHAPTER 1

ECOLOGY OF THE CULTURE PONDS AND

OBSERVATIONS ON WHITE SPOT SYNDROME (WSS)

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ECOLOGY OF THE CULTURE PONDS AND OBSERVATIONS ON WHITE SPOT SYNDROME (WSS)

1.1 INTRODUCTION

Intensive mariculture has been receiving worldwide attention as an alternative to depleted fishery resources and the declining profitability of industrial fishing. Stagnation in the prawn landings could be seen in recent years, despite increasing fishing effort. Prawn culture being the world's most rapidly expanding warm water aquaculture sector (Phillips *et al.*, 1993), it is not surprising that, of all forms of aquaculture activities, shrimp culture has received the greatest attention from researchers, planners and developers in recent years. In the tropics, shrimp mariculture is based on two genera, *Penaeus* and *Metapenaeus,* the former being by far the more predominant group. Currently six species, five penaeids and one metapenaeid are cultured in the tropics. *Penaeus monodon* is the most popular, accounting for about 65% of the tropical shrimp production and occurring mainly in Asia (Chen *et al.*, 1989b; Rosenberry, 1991; Csavas, 1993, 1994). *P. vannamei,* followed by *P. stylirostris* are the predominant species cultured in South American countries, principally, Ecuador, Colombia, etc. In India, of the four species of cultivable marine prawns, i.e., *P. monodon, P. indicus, P. merguiensis* and *P. semisulcatus,* the former two are cultured mainly. During the last decade, prawn culture has expanded in India, both in terms of area (85,000 ha) and yield (1,00,000 t) (Achuthankutty *et al.*, 1998).

Prawn farming can be classified into extensive, semi-intensive, intensive and super or ultra intensive culture systems, on the basis of the intensity

of culture operations. The former two are greatly practised in India. Eventhough, recognition, prevention and treatment of disease is possible in most semi-intensive and all intensive culture systems, it is the very nature of these two culture systems (i.e., high shrimp density per unit volume of water used) that encourages the development and transmission of many diseases (Lightner, 1993). Water quality management is one of the key factors in aquaculture. According to Flegel *et al.* (1995), the most significant management error in causing diseases was postlarval stocking beyond the carrying capacity of individual ponds. In an aquatic environment, physical and chemical factors like, temperature, salinity, dissolved oxygen, pH, alkalinity, nutrients and reducing gases like hydrogen sulphide would exercise their influence individually or synergistically. Most environmental stress is multi-factorial (Flegel *et al.*, 1995).

According to Roberts (1978), probably, the most important stress factor, affecting the balance between the fish host and the environment is environmental temperature. As the temperature approaches the maximum and minimum extremes, which are specific for each species, pathogenic invasion is likely to occur, especially at the maximum temperature levels (Roberts, 1978). Even within the acceptable normal temperature range, temperature could be significant as a cause of disease. Alterations in temperature might affect the rate of multiplication of microorganisms, the amount of dissolved oxygen in the water, the rate of excretion of metabolites or, most importantly, the speed with which the host's defensive mechanisms could develop. Survival rates of postlarvae and juveniles of *P. monodon* were found to be generally lower at 33 °C. As water temperature declined, growth eventually ceased (Wheeler, 1968b; More and Elam, 1970; Lumare and Villani, 1972; Tournier, 1972), and as it declined further, feeding stopped (Liao, 1969; Shigueno, 1972). The effects of

temperature on the growth and survival of prawns in ponds have been studied by Venkataramiah *et al.* (1974), Menz and Bowers (1980), Rubino *et al.* (1983), Aquacop (1984), Seidman and Issar (1988), Wyban and Sweeney (1989) and Padlan (1990). Temperature has been identified as one of the factors, having a profound effect on adaptive mechanisms of crustaceans to salinity (Mantel and Farmer, 1983).

When prawns are exposed to salinity higher or lower than its optimum, it must osmoregulate to maintain the internal salt balance. For this, more energy is required, which critically affects their normal growth. The salinity of the brackishwater pond generally depends on the salinity of the adjacent estuary. According to Balakrishnan (1957) and George and Kartha (1963), the salinity of an estuary generally is that of freshwater during monsoon and rises near to that of seawater during extreme summer months.

Dissolved oxygen exerts a tremendous effect on growth and production through indirect effect on metabolism and feed consumption and direct effect on environmental conditions. A dissolved oxygen content, above 3.5 ppm was required for the proper growth of prawns (Suseelan, 1978). Higher rate of organic decomposition and heavy phytoplankton blooms reduced the dissolved oxygen content to critical levels during early morning hours. Dissolved oxygen level, below 2 ppm has been considered as a stress factor (Kramer, 1975; Seidman and Lawrence, 1985; Liao and Murray, 1986; Padlan, 1990). Low dissolved oxygen concentrations increased the toxicity of ammonia to fish (Alabaster *et al.*, 1979; Thurston *et al.*, 1981). Lloyd (1961) attributed this phenomenon to an increase in the uptake of ammonia as gill ventilation rates increased to prevent hypoxia.

pH is a measure of hydrogen ion concentration in water and it indicates the rate of acidity or alkalinity of water. Guo and Xu (1994) gave the general trend of water pH in prawn culture ponds. According to Muthu (1980) and Tsai (1990), brackishwater with pH ranging from 7.5 to 8.5 was conducive for the culture of prawns. Low pH could cause damage to gill tissues of fish (Ferguson, 1988) and could also influence the impact of potential toxins like ammonia (Alabaster and Lloyd, 1980; Colt and Armstrong, 1981) and heavy metals (Boyd, 1989). Low pH could also reduce natural pond productivity by reducing the availability of nutrients (Alabaster and Lloyd, 1980), including phosphorus (Boyd, 1982) and carbon sources for photosynthesis (Allan and Maguire, 1992). The effect of pH and ammonia on survival and growth of the larval stages of *P. monodon* was studied by Bower and Bidwell (1978), Boyd (1982) and in recirculating system, following nitrification (Wickins, 1983). The coastal areas in many South East Asian countries were reported to suffer acid sulphate soils (Porter, 1976; Simpson and Pedini, 1985). Poor growth and mortality of penaeids occurred when acid sulphate soils acidify pond waters, during periods of heavy rains (Webber and Webber, 1978; Apud *et al.*, 1985; Mrithunjayan and Thampy, 1986). In such cases, decreased salinity might accompany decreased pH. In acid waters, crustaceans and fish might experience impaired ionic regulation (Morgan and McMahon, 1982; Havas and Hutchinson, 1983; Hobe *et al.*, 1983, 1984).

Nitrogen enters the culture system, primarily in the organic form and metabolises to ammonia, nitrite and nitrate, by the activity of either the resident organisms or by bacteria (Armstrong, 1979; Jhingran, 1982). Ammonia was the principal nitrogenous product excreted by crustaceans (Hartenstein, 1970;

Kinne, 1976; Claybrook, 1983; Regnault, 1987) and might accumulate in culture systems, followed by microbial decomposition of organic material (Stanier *et al.*, 1976) and with some fertilisation practices (Boyd, 1982). In penaeid prawns, ammonia might affect the acid-base balance, haemolymph osmolarity, nitrogen metabolism, respiration, growth rate, might enhance moulting and in extreme cases, result in mortality (Wickins, 1976; Armstrong *et al.*, 1978; Chen and Kuo, 1992; Chen and Lai, 1992; Chen and Cheng, 1993; Robles, 1997). Ammonia was believed to cause damage to central nervous system (Wright, 1995). In solution total ammonia comprised unionised (NH3) and ionised ammonia (NH4) in equilibrium and their proportion depended on pH, temperature and salinity (Trussell, 1972; Whitfield, 1974, 1978). Unionised ammonia was more toxic (Smart, 1978), the concentration of which was more at basic pH. Ionised ammonia might also become toxic, especially at low pH levels (Shaw, 1960; Armstrong *et al.*, 1978).

Ammonia was oxidised to nitrite and nitrate by nitrification (Sharma and Ahlert, 1977). Ammonia and nitrite were the most common toxicants, which could limit production in intensive and semi-intensive crustacean aquaculture (Delistraty *et al.*, 1977; Thurston *et al.*, 1978; Colt and Armstrong, 1981). The effect of ammonia on penaeid prawns had been elaborately studied by Catedral *et al.* (1977), Jayasankar and Muthu (1983a, 1983b), Carpenter *et al.* (1986), Chin and Chen (1987), Wyban *et al.* (1987), Chen and Chin (1988), Chen *et al.* (1989a), Courtney (1989), Wajsbrot *et al.* (1989), Allan *et al.* (1990), Chen and Lei (1990), Chen and Wang (1990), Chen and Kou (1993) and Alcaraz *et al.* (1999a, 1999b).

Nitrite was an intermediate product in the metabolism of ammonia and was highly toxic at decreased pH, because the relative proportion of toxic unionised nitrous acid increased (Colt and Tchobanoglous, 1976; Russo *et al.*, 1981). Cole and Boyd (1986) had shown that nitrite concentration increased with increased feeding rate. Nitrite also had been found to oxidise the respiratory pigment (Needham, 1961). Armstrong (1979) and Chen *et al.* (1986) opined that nitrite was more toxic than ammonia. Previous studies on the toxicity of nitrite to crustacean larvae had involved lobsters, *Homarus americanus* (Delistraty *et al.*, 1977), freshwater prawns, *Macrobrachium rosenbergii* (Armstrong *et al.*, 1978) and penaeids (Jayasankar and Muthu, 1983a, 1983b; Chin and Chen, 1987; Chen and Chin, 1988; Allan *et al.*, 1990; Chen and Lei, 1990; Chen and Kou, 1993; Alcaraz *et al.*, 1999a, 1999b).

Nitrate was the least toxic form of nitrogen in the culture system (Chen *et al.*, 1990). According to Gopinathan *et al.* (1982), the nitrate values were moderate to high in brackishwater ponds located at the middle portion of the Cochin estuarine system, while those in the northern and southern ends were lower. This is an essential nutrient for primary production. Mathews (1992) observed higher nitrate concentration at the middle (Narakkal) and northern ends (Cherai) of Cochin estuarine system.

Phosphate was found to be the most critical single factor in the maintenance of pond fertility (Mandal, 1980). This was due to the fact that phosphorus is very much essential in the operation of energy transfer systems of the cell and it normally occurs in very small quantities (Reid and Wood, 1976). Phosphorus in natural waters occurred in three different forms, namely, inorganic or soluble phosphate-phosphorus, insoluble, organic phosphorus and

the particulate organic phosphorus. Phosphate content was found to vary widely between the ponds at the middle and northern ends of the Cochin estuary, though, the values were generally high in these ponds. According to Boyd (1982), lack of phosphorus and nitrogen limited phytoplankton productivity especially in brackishwater ponds. The importance of increasing the natural level of nutrients in water, especially the role of phosphate in the trophic chains had been discussed by Seymour (1980).

The main source of sediment in the culture system is soil eroded from the pond walls and from the periphery of the pond bottoms. Inputs of food, faeces and decaying plankton enrich the sediment, increasing the organic carbon content. Soil is generally considered as the "laboratory of the pond" because many chemical reactions are governed by soil quality parameters (Hickling, 1971). Sediment has a considerable buffering capacity on the quality of the water layer above it. It provides water with nutrients and serves as a biological filter through the adsorption of the organic residues of food, body excretions and algal metabolites (Chen *et al.*, 1989b).

Shrimp pond soils varied greatly in pH (Boyd, 1992). The pH decreased in all pond soils owing to the oxidation of organic matter and/or inorganic compounds. Highly acidic conditions, as observed in acid sulphate soils, impeded organic matter decomposition and recycling of nutrients, decreased the availability of phosphate, and had direct inhibitory effects on benthos and shrimps (Boyd, 1992).

Brackish water pond soil was found to be poor in organic carbon content (Chakraborti *et al.*, 1985). Pond management between crops might be

improved by knowing the percent of organic carbon in the bottom soil, as high levels of organic matter (OM) indicated deteriorating conditions. The organic matter content in bottom soil increased with increasing water depth (Boyd, 1977). According to Tang and Chen (1967) and Chakraborti *et al.* (1985), increase in organic carbon content increased the yield. Very low organic content was reported by Gopinathan *et al.* (1982) in brackishwater ponds off Cochin estuarine system and by Rajyalakshmi *et al.* (1988) in brackishwater ponds off Chilka lake. However, Eswaraprasad (1982) and Ramesan (1990) reported higher values of organic carbon in the brackishwater ponds off Cochin estuarine system.

Shrimp pond water is high in nutrients, solids, BOD and has high levels of pathogenic bacteria and dissolved organic matter, the concentration of which are related to the feeding rate and other pond management criteria (Hopkins *et al.*, 1995; Smith, 1996). It is the case with pond sediment also. When discharged, this water and sediment might enhance eutrophication in the adjacent estuary or near shore ecosystems. In such a situation, phytoplankton always greatly bloomed, diversity index was lowered and bacteria or virus developed fast. Therefore, optimising feed management and maximising use of natural food production were critical for economic survival of farms in many areas of the world, particularly in view of decreasing profit margins, brought about by recent developments, especially viral disease outbreaks and other problems (Jory, 1998).

Major disease losses, experienced during rearing of black tiger prawn, *Penaeus monodon*, in Thailand were caused either by pond management errors or external environmental factors that influenced the rearing pond

environment (Flegel *et al.*, 1995). According to Flegel *et al.* (1995), successful cultivation depended on maintaining pond salinity, pH and dissolved oxygen in appropriate ranges. Experimental infection trials carried out by Wang *et al.* (1997b) showed that environmental stress, such as ammonia might enhance the severity of WSD virus infections in cultured shrimp. According to Kongkeo (1997), high water exchange systems in Indonesia and Philippines introduced viruses, other pathogens, excess organic loads, ammonia and other toxic particles released by nearby farms, through the incoming water. Despite serious crop failures in other countries, within the past few years, the annual shrimp production in Thailand still remained high, because farmers had readily adopted new, environmentally friendly and locally suitable, water exchange systems, such as less water exchange and closed, full strength seawater and freshwater systems, overcoming viral and other disease infections (Anon, 1995; Kongkeo, 1997). Based on an experiment conducted in the intensively stocked *Penaeus vannamei* ponds, Hopkins *et al.* (1995) opined that intensive shrimp farming might be possible in static, no-exchange systems, thereby minimising the potential impact of effluent, as long as feed inputs did not overwhelm the assimilative capacity of the ecosystem. According to Fegan *et al.* (1991), the viral disease was well tolerated by *P. monodon*, so long as rearing conditions were optimal.

Prior to WSSV outbreak in India, during 1994-1995 period, there was a severe shortage of seeds in India (Alagarswami, 1995). One argument was that the virus came to India, through clandestine import of nauplii and breeders. Since the farmers simply drained the infected ponds, the virus seemed to have infected the natural stock of penaeid prawns and crabs. Thus, there seemed to be a natural reservoir of infection and this was a continuing problem

in India (Karunasagar *et al.*, 1998). In the east coast of India, WSS epizootic occurred immediately after the cyclone and rains (Anonymous, 1995). Even, along the west coast, the outbreak occurred when the monsoon was at its peak, salinity at undetectable low levels, with heavy surface run-off and turbidity in natural waters. These observations supported the view that environmental factors were important in triggering an epizootic of WSS (Karunasagar *et al.*, 1997).

Eventhough, the various ecological parameters in prawn culture ponds had been studied in detail in India and abroad, information regarding the range of these parameters in culture ponds at the time of occurrence of WSD was very scanty. In the present study, a sincere attempt has been made to elucidate whether the various ecological parameters recorded in culture ponds, where WSD appeared were within the tolerable limits or not. The symptoms of WSD and the vulnerable size ranges of various prawn species were also recorded.

1.2 MATERIALS AND METHODS

DESCRIPTION OF THE COLLECTION PONDS

Three sites along the Cochin backwaters, at Narakkal, Pallipuram and Chellanam were located for the present study (Fig. 1). The selection criterion for the ponds in these areas was the previous occurrence and non-occurrence of the epizootic, white spot syndrome (WSS). The perennial pond at Narakkal, where prawn farming was practised and which was known to be

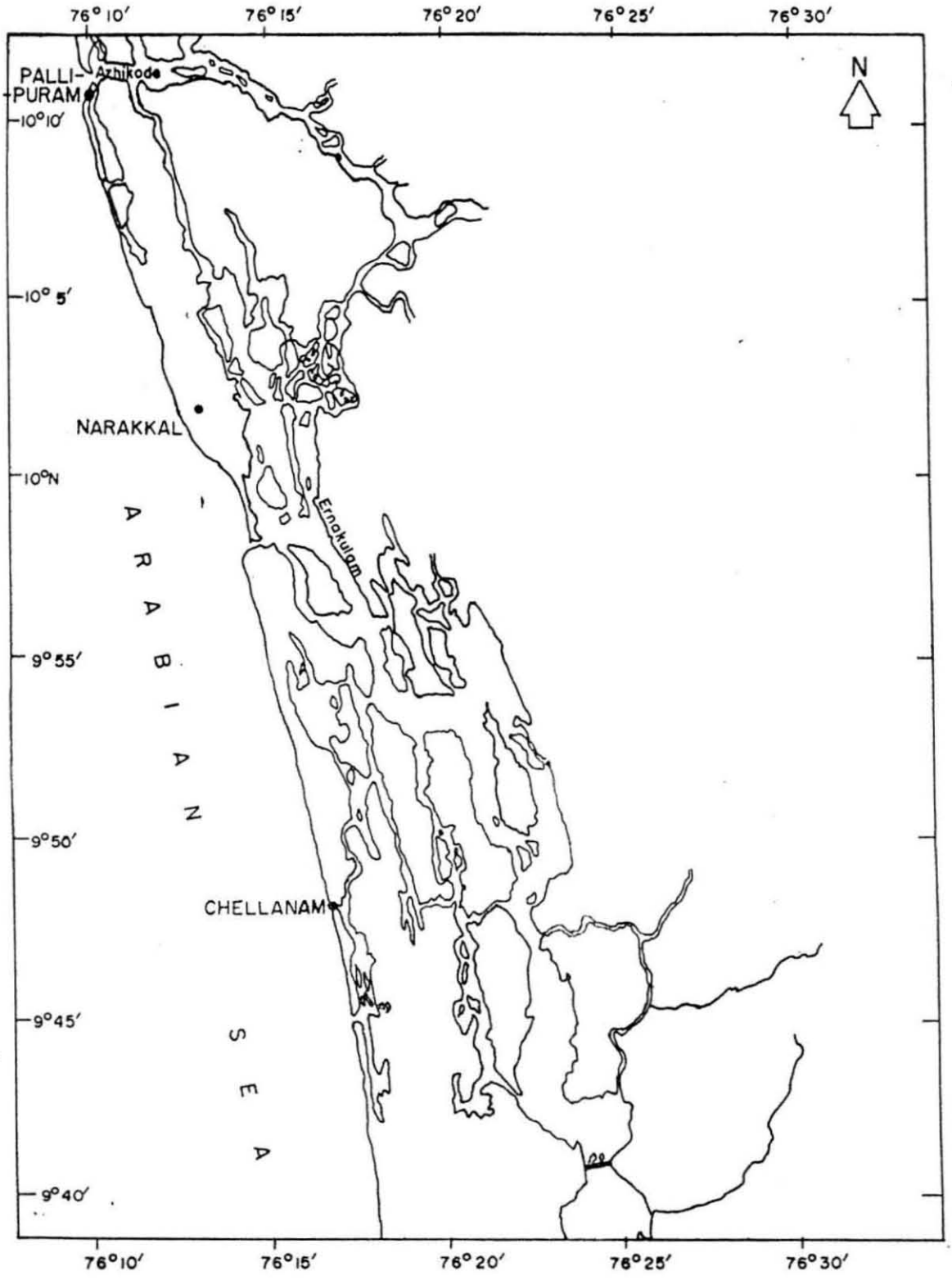


Fig. 1. Map showing areas of study and station positions.

absolutely free from any viral disease outbreak so far was chosen as the control pond N (Fig. 6). Two prawn culture ponds, pond P_1 and pond P_2, which had a history of disease outbreak were selected at Pallipuram (Figs. 2 & 3) and two disease free ponds, pond C_1 and pond C_2 were selected at Chellanam (Figs. 4 & 5) for the present study. The depth of the ponds ranged from 0.8m to 1.0m. The period of study extended from November 1997 to March 1999 and it included four crops (1997 November- 1998 January, 1998 March- 1998 May, 1998 August- 1998 October and 1999 January- 1999 March). Samples were collected continuously from all the five ponds for the study of the major ecological parameters.

The area of the ponds, P_1, P_2, C_1, C_2 and pond N were 0.5 ha, 1.0 ha, 0.21 ha, 1.2 ha and 0.2 ha respectively and were stocked with postlarvae-18 to PL-20 of both *Penaeus indicus* and *P. monodon* seeds at the rate of 10 numbers/m^2. Hatchery produced seeds were used for the purpose. The pond preparation before stocking included eradication of wild fish and other predatory organism, by continuous netting, application of lime and drying. Aeration was provided by operating paddle wheel aerators and tidal exchange. The prawns were fed at the rate of 8% of their body weight with "Higashi starter" and thereafter "Higashi grower" was used as feed.

Samples of water, soil and prawns were collected once in every fortnight from each pond, between 07.00 and 08.00 Hrs in the morning. Water and soil samples were collected in duplicates and the average value for each hydrographical and soil quality parameter was calculated. Water samples were taken in sterile plastic bottles and soil samples were collected in sterile plastic covers and brought to the laboratory in iceboxes. Diurnal variation in

Fig. 2. Pond P_1 at Pallipuram

Fig. 3. Pond P_2 at Pallipuram

Fig. 4. Pond C_1 at Chellanam

Fig. 5. Pond C_2 at Chellanam

Fig. 6. Pond N at Narakkal

Fig. 7. Portion of a catch from pond C_1

temperature and dissolved oxygen were also recorded in pond C_1, C_2 and pond N. Prawn samples were cast nested from different sites of the ponds and processed for histological and ultrastructural examination, as described in chapters 2 and 3.

WATER QUALITY PARAMETERS

1) TEMPERATURE

Temperature of the water was measured at the pond site itself by using a 0-100 °C high precision thermometer.

2) SALINITY

For the estimation of salinity of the water sample, Mohr titration method was used, as indicated by Strickland and Parsons (1972). 10 ml of the sample was titrated against standard silver nitrate solution, using potassium chromate as the indicator. Salinity was calculated using the formula,

$$\text{Salinity of sample (‰)} = \frac{V1 \times S}{V2}$$

, where, 'V1' and 'V2' are the volume of silver nitrate used for titrating 10 ml each of the sample and standard seawater respectively and 'S' is the salinity of the standard seawater (35 ppt).

3) DISSOLVED OXYGEN

Dissolved oxygen was estimated by Winkler method modified by Carrit and Carpenter (1966). The outline of the method is as follows (FAO, 1975).

Water samples for estimating dissolved oxygen were taken carefully in 125 ml, BOD bottles, without any air bubbles and fixed immediately by adding 1 ml each of Winkler A and Winkler B solutions. The solution was mixed thoroughly and the precipitate was allowed to settle. Later this precipitate was dissolved completely by shaking well with 2 ml concentrated hydrochloric acid. From this, 20 ml was transferred to a 250 ml conical flask and was titrated against standard sodium thiosulphate solution to a pale straw colour. 1 ml of starch solution was added as an indicator, which imparted a deep blue colour to the sample. Titration was continued until the blue colour disappeared. Concentration of dissolved oxygen was calculated using the formula,

$$DO\ (ml/l) = \frac{V1 \times N \times 8 \times 1000 \times R}{V_2 \times 1.429}$$ where,

V_1 = volume of Sodium thiosulphate

N = normality of Sodium thiosulphate

V_2 = volume of water sample taken for titration against the Sodium thiosulphate

R = correction factor equal to 1.01.

4) WATER pH

pH of the water sample was measured in the laboratory by an Elico pH meter model L1-120, having a combination electrode. The instrument was calibrated with pH buffers 4, 7 and 9.2.

5) NUTRIENTS

a) Ammonia-nitrogen

The method followed for the determination of ammonia in seawater is that of Zolarzano (1969) and it involved indo-phenol blue reaction.

The procedure consisted of the successive addition of 2 ml of phenol solution, 2 ml of sodium nitroprusside solution and 5 ml of oxidising reagent to 50 ml of the sample, mixing thoroughly after each addition. The colour is allowed to develop at room temperature for one hour, and the absorbance is recorded at a wave length of 640 nm in a spectrophotometer (Spectronic 1001). Standard ammonia solution was prepared at different concentrations and the absorbance was noted. Sample concentration (ppm) was recorded from the standard graph.

b) Nitrite-nitrogen

Estimation of nitrite nitrogen was done by the method devised by Morris and Riley and described by Strickland and Parsons (1972).

50 ml of the seawater sample was taken in a clean conical flask and to it was added 1 ml of the sulphanilamide solution. After two minutes, 1 ml of NNED solution was added and mixed immediately. Absorbance of the solution was measured at 545 nm and the concentration of the sample was read from the standard graph, drawn with the absorbance of the standard nitrite solution.

c) Nitrate-nitrogen

Nitrate nitrogen was estimated following the method of Morris and Riley and modified by Strickland and Parsons (1972).

To 50 ml of the water sample, 2 ml of the buffer reagent (phenol and sodium hydroxide) was added and with rapid mixing, 1 ml of the reducing agent (copper sulphate and hydrazine sulphate) was also added. The sample was kept away from sun light in a dark place for about 20 hours. 2 ml of acetone was added, followed by 1 ml of sulphanilamide solution, after 2 minutes. After another two minutes, 1 ml of NNED was also added and mixed well. The absorbance was measured at a wavelength of 545 nm and the nitrate nitrogen concentration in the sample was recorded from the standard graph, prepared by plotting the absorbance of the standard nitrate solution at various concentrations.

d) Phosphate-phosphorus

The method given by Murpny and Riley and described by Strickland and Parsons (1972) was used for the determination of reactive phosphorus.

To 50 ml of the water sample was added 5 ml of the mixed reagent (ammonium molybdate solution, sulphuric acid solution, ascorbic acid and potassium antimony tartarate). A blue colour was allowed to develop. After five minutes, and preferably within the first 2 to 3 hours, the absorbance of the solution was measured at 885 nm in a spectrophotometer. Standard graph was drawn with different concentrations of anhydrous potassium dihydrogen phosphate and the sample concentration was recorded from it.

SOIL QUALITY PARAMETERS

Soil samples were collected in duplicates and brought to the laboratory by keeping them in the icebox. Wet samples were used for pH determination and afterwards, the soil samples were dried. The samples were then powdered, sieved and stored in sterile polythene bags, for the analysis of organic carbon.

i) Sediment pH

pH of the sediment sample was determined in the wet condition by using an Elico digital pH meter.

ii) Organic carbon

The method followed for the determination of organic carbon was the chromic acid method of Walkley and Black (1965).

1 g of the powdered soil sample was taken in a clean, dried 250 ml conical flask. 10 ml of 1 N dichromate solution was added to this and swirled for about ten minutes for proper mixing. After this, 20 ml of concentrated sulphuric acid containing 1.25% silver sulphate was added to the sample, swirled well and allowed to react for about 30 minutes. The sample was then diluted to 200 ml with distilled water and 10 ml concentrated orthophosphoric acid was added to this. After a thorough mixing, 1 ml of diphenylamine indicator was added and the solution was titrated with 0.4 N ferrous ammonium sulphate solution. The end point was indicated by a sharp colour change from blue to brilliant green. Along with the sample, a blank was also run. The percentage of organic carbon in the soil sample was calculated using the formula,

Organic carbon (%) = $\frac{3.95}{g}$ (1- $\frac{T}{S}$), where, 'T' and 'S' are the volume of ferrous ammonium sulphate used for sample and blank respectively and 'g' indicates the weight of the soil sample in grams.

Specimens of *Penaeus indicus*, *P. monodon* and *Metapenaeus dobsoni* were collected from ponds at Chellanam, where white spot disease (WSD) appeared. Their length and weight measurements were taken sex wise. 92 specimens of *P. indicus* males and 101 numbers of *P. indicus* females were measured. Length was measured from tip of the rostrum to the tip of the telson. 210 numbers of *P. monodon* males and 217 females were considered. For *M. dobsoni*, 61 females and 83 males were collected from the affected ponds. All the specimens were examined thoroughly under a dissection microscope. For measuring weight, the specimens were kept on blotting paper for removing water for a fixed time period and weight was taken by using an electronic digital balance of the model, Sartorius.

For the experimental studies, apparently healthy specimens of *Penaeus indicus*, measuring 70-100 mm in length were brought to the laboratory in the oxygen packs. They were acclimatised in the cement tanks of 100 *l* capacity. They were divided into three groups of 16 animals each and reared for 2 days in separate tanks with regular water exchange, aeration and fed with minced healthy prawn meat. After 2 days, the animals in tank 1 were given minced meat of WSSV affected *P. monodon*, collected from ponds P_1 and P_2 at Pallipuram. Regular aeration and water exchange were given. In tank 2 and 3, the animals were fed with 6 % of their body weight with minced meat of healthy penaeid prawns. In tank 2, the animals were kept in the mud and water

brought from WSD affected ponds P_1 and P_2 and optimum aeration was given without much water exchange. Only 20 % of the water was exchanged daily in tank 2. Regular water exchange (90%) and feeding with normal, healthy prawn meat were continued in tank 3. The experiment was run in triplicates.

1.3 **RESULTS**

The results obtained for water quality parameters, such as, temperature, salinity, dissolved oxygen, water pH, ammonia, nitrite, nitrate, phosphate and two soil quality parameters, namely, soil pH and organic carbon content from culture ponds, P_1, P_2, C_1, C_2 and pond N are given below.

A) ECOLOGICAL PARAMETERS IN CULTURE PONDS

WATER QUALITY PARAMETERS

1. WATER TEMPERATURE

In the beginning of culture in November 1997, water temperatures recorded in pond P_1 and P_2 were 30.06 °C and 29.75 °C respectively. Thereafter, a marginal increase in temperature was recorded till March 1998 and the values were comparable in both the ponds. An increase in temperature was observed in ponds P_1 and P_2 in March 1998 and the respective values recorded were 32.8 °C and 32.05 °C. A maximum of 33.1 °C and 33.0 °C were recorded in ponds P_1 and P_2 in May 1998. A sharp decline in temperature was observed in June 1998, reaching 31.05 °C and 31.1 °C respectively. In July and August 1998 water temperature declined gradually. An increasing trend was observed in September and October 1998. Thereafter, the temperature showed

slight fluctuation in both the ponds and in March 1999, the recorded values were 32.05 °C and 32.25 °C respectively in ponds P_1 and P_2 (Table 1 and fig. 8).

Water temperature observed in ponds C_1 and C_2 showed very little variation and in both the ponds, temperature gradually increased from November 1997 to May 1998. The range reported in ponds C_1 and C_2 were from 28.09 °C to 30 °C and 28.05 °C to 30.8 °C respectively. Peak temperature was recorded in May 1998, the values being 30.0 °C and 30.8 °C in ponds C_1 and C_2 respectively. Thereafter, a sharp decline was observed in both the ponds till August 1998, followed by a marginal increase up to November 1998. Again, a sharp decrease in temperature was reported in pond C_1 from 28.8 °C in November 1998 to 27.5 °C in December 1998 and a marginal increase was followed in the last two months. In pond C_2, a gradual increase in temperature was observed till February 1999, the increase being from 28.0 °C in December 1998 to 29.0 °C in February 1999 (Fig. 8).

Compared to the above ponds P_1, P_2, C_1 and C_2, an overall increase in temperature was observed in pond N. In the beginning of culture in November 1997, the recorded temperature in pond N was 31.65 °C and thereafter, a marginal increase was observed up to May 1998. The maximum recorded temperature, 34.0 °C was observed in May 1998, followed by a sharp decline to 31.5 °C in June. The minimum temperature, 28.05 °C was reached in August 1998 and thereafter, a marginal increase was observed till February 1999, the last month of study. The general trend observed in monthly variation of water temperature in all the five ponds during the present study period are represented in table 1 and figure 8.

Table 1. **Monthly variations in water temperature (°C) in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	30.06	29.75	28.09	28.05	31.65
Dec	30.55	30.15	28.01	28.08	31.6
98 Jan	30.93	30.4	28.94	29.4	31.75
Feb	31	30.5	29.29	29.65	31.85
Mar	32.8	32.05	29.63	29.9	33.25
Apr	32.9	32.5	29.91	30.5	33.85
May	33.1	33	30	30.8	34
Jun	31.05	31.1	29	29.5	31.5
Jul	29	29.2	28	28.2	29
Aug	28.65	29	27.75	27.1	28.05
Sep	29.75	30.2	28.28	27.4	28.15
Oct	30	30.35	28.75	28.18	29.5
Nov	29.75	29.4	28.8	28.4	30.4
Dec	31.93	30.77	27.5	28	30.7
99 Jan	30.8	30	28.11	28.12	31.1
Feb	31	30.5	28.4	29	32
Mar	32.05	32.25	29.38		

Table 2. **Diurnal variations in water temperature (°C) in ponds at Chellanam (C_1 & C_2) and Narakkal (N)**

Time	Pond C_1	Pond C_2	Pond N
6:00 AM	29.9	30.8	30
9:00 AM	30.5	31.5	30.4
12 Noon	34.6	34.4	34.9
3:00 PM	35.75	35.8	35.6
6:00 PM	35.25	35.2	34.5
9:00 PM	34.4	34	33.2
12 Night	33.2	33.5	32.8
3:00 AM	31.8	32	32
6:00 AM	30	30.5	30.6

Fig. 8. Monthly variation in water temperature in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)

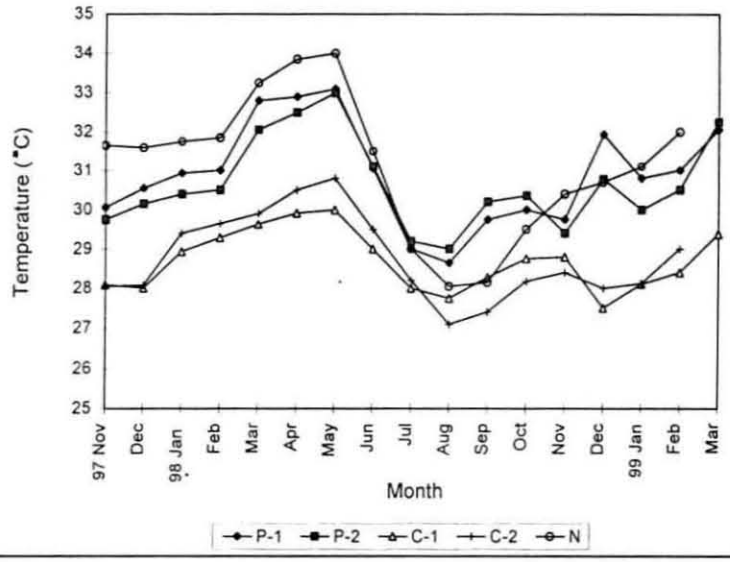
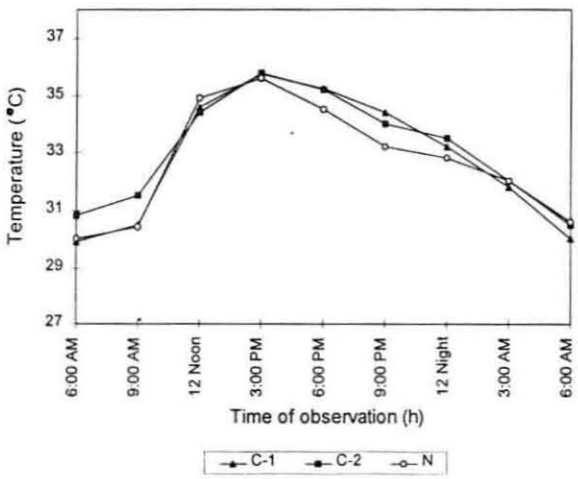


Fig. 9. Diurnal variation in water temperature in ponds at Chellanam (C_1 & C_2) and Narakkal (N)



A diurnal variation in temperature, recorded in three ponds, pond C_1, C_2 and pond N are given in figure 9. At 06.00 A.M., the temperature was minimum and the values for ponds, C_1, C_2 and N being, 29.9 °C, 30.8 °C and 30.4 °C respectively. During daytime, the temperature showed a sharp increase and the maximum values of 35.75 °C, 35.8 °C and 35.6 °C were recorded respectively at 03.00 P.M. Thereafter, the temperature declined gradually, reaching the lowest value of 30.0 °C, 30.5 °C and 30.6 °C respectively in ponds C1, C2 and N at 06.00 A.M. (Table 2 and fig. 9).

2. SALINITY

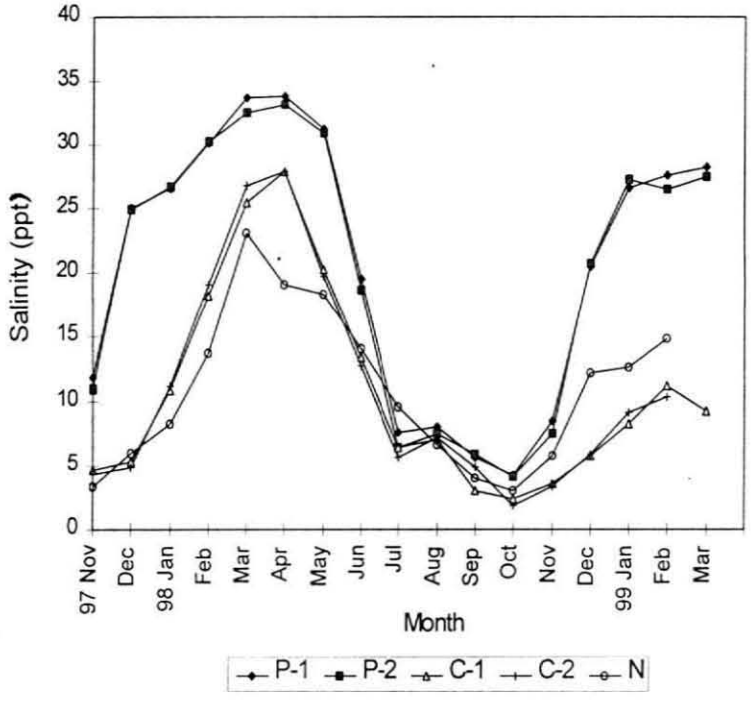
Salinity of water recorded in ponds P_1 and P_2 were comparable. In November 1997, salinity observed in ponds P_1 and P_2 were 11.86 ppt and 11.0 ppt respectively. This was followed by a sudden increase in December 1997 and the corresponding values being, 25.0 ppt and 24.9 ppt. Thereafter, marginal increases in salinity were observed in both the ponds till April 1998, the maximum-recorded values being 33.77 ppt and 33.13 ppt respectively. From May 1998 onwards, a sharp decline in salinity was observed till July 1998, followed by a trend of fluctuation up to November 1998 in both the ponds. Again, in December 1998, a sharp rise in salinity was observed, recording 20.41 ppt and 20.7 ppt in ponds P_1 and P_2 respectively, followed by a marginal increase till March 1999 (Table 3 and figure 10).

An almost same trend in salinity of water was recorded in ponds C_1 and C_2. The values were 4.69 ppt and 4.33 ppt respectively in November 1997, followed by a sharp increase up to April 1998. The maximum salinity recorded

Table 3. **Monthly variations in salinity (ppt) in water recorded in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	11.86	11	4.69	4.33	3.34
Dec	25	24.9	5.32	4.87	5.96
98 Jan	26.51	26.67	10.83	11.23	8.3
Feb	30.16	30.25	18.16	19	13.72
Mar	33.73	32.49	25.48	26.77	23.08
Apr	33.77	33.13	27.88	27.88	19.04
May	31.23	30.83	20.23	19.7	18.31
Jun	19.45	18.63	13.33	12.67	14.07
Jul	7.65	6.42	6.42	5.63	9.59
Aug	8.06	7.46	7.07	7.13	6.65
Sep	5.6	5.89	2.99	4.85	3.99
Oct	4.19	4.11	2.41	1.82	3.07
Nov	8.5	7.51	3.57	3.32	5.81
Dec	20.41	20.7	5.81	5.89	12.19
99 Jan	26.54	27.2	8.29	9.12	12.61
Feb	27.53	26.37	11.2	10.3	14.76
Mar	28.2	27.4	9.29		

Fig. 10. Monthly variation in salinity in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)



in ponds C_1 and C_2 were in April 1998, the values being 27.88 ppt in each pond. Thereafter, a sharp decline (from 19.7 ppt to 5.63 ppt) was observed till July 1998. This was followed by a fluctuation in both the ponds up to November 1998. From December 1998 onwards, gradual rise in salinity were observed in ponds C_1 and C_2. In pond C_1, a sudden drop in salinity (from 11.2 ppt to 9.29 ppt) was recorded in March 1999 (Table 3 and fig. 10).

In pond N, a salinity of 3.34 ppt was recorded in the beginning of culture in November 1997, followed by a sharp rise up to March 1998. The maximum recorded salinity in pond N was 23.08 ppt in March 1998 and thereafter, a gradual decline was observed till October 1998, in which month, the lowest recorded salinity of 3.07 ppt was noticed. From November 1998 onwards, a marginal rise in salinity was observed in pond N, reaching 14.76 ppt in February 1999. The general pattern of monthly variation in salinity recorded in all the five ponds were shown in table 3 and figure 10.

3. DISSOLVED OXYGEN

In the beginning of culture (November 1997), the dissolved oxygen observed in ponds P_1 and P_2 were 4.18 and 4.78 ml/l respectively. This was followed by a sharp decline in December 1997 and January 1998. Again in February 1998, dissolved oxygen concentration increased in both the ponds, the increase being from 2.93 and 2.91 ml/l in January 1998 to 3.8 and 3.79 ml/l in February 1998 in ponds P1 and P_2 respectively. Thereafter, the values declined sharply to 2.69 and 2.92 ml/l in ponds P_1 and P_2 respectively in April 1998 and a sudden increase was observed from May 1998 to August 1998 (Table 4 and figure 11). Maximum dissolved oxygen concentration of 4.72 ml/l and 4.5 ml/l were recorded in ponds P_1 and P_2 respectively in July 1998. From September

1998 onwards, till the end of the present study period in March 1999, a gradual decline in dissolved oxygen was observed in both the ponds P_1 and P_2.

In ponds C_1 and C_2, the recorded dissolved oxygen concentration in November 1997 were 3.77 ml/l and 3.93 ml/l respectively, followed by fluctuation in dissolved oxygen till May 1998. Thereafter, an increase of 3.68 ml/l and 3.36 ml/l in June 1998 and the maximum concentrations of 4.09 ml/l and 4.76 ml/l in August 1998 were observed in ponds C_1 and C_2 respectively. This was followed by a fluctuation in dissolved oxygen concentration in pond C_1. In pond C_2, a marginal decline was observed, the minimum dissolved oxygen concentration being 1.18 ml/l in March 1999 (Figure 11).

Compared to the above reported dissolved oxygen concentration, slightly lower values were recorded in pond N, the initial dissolved oxygen concentration being, 2.72 ml/l in November 1997. Thereafter, the dissolved oxygen reached the lower concentration of 1.86 ml/l in January 1998, followed by a fluctuation in dissolved oxygen till the end of culture in February 1999. The maximum dissolved oxygen recorded was 3.76 ml/l in August 1998 and the minimum concentration 1.64 ml/l in November 1998 (Table 4 and fig. 11).

Diurnal variation in dissolved oxygen were recorded in ponds C_1, C_2 and N, at the time of occurrence of white spot disease in the former two ponds. The lowest dissolved oxygen concentration were observed at 06.00 A.M. and the highest at 06.00 P.M. in all the three ponds. The dissolved oxygen concentration in ponds C_1, C_2 and N at 06.00 A.M. were, 1.67 ml/l, 0.5 ml/l and 0.82 ml/l respectively on the first day of observation and 0.79 ml/l, 0.55 ml/l and 0.96 ml/l respectively on the second day. The dissolved oxygen concentration marginally increased till 06.00 P.M. in ponds C_1 and C_2 and a

Table 4. **Monthly variations in dissolved oxygen (ml/l) in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	4.18	4.78	3.77	3.93	2.72
Dec	2.61	3.16	2.88	2.94	2.45
98 Jan	2.93	2.91	2.92	2.49	1.86
Feb	3.8	3.79	3.07	2.88	2.16
Mar	3.27	3.19	3.22	3.26	3.05
Apr	2.69	2.92	3.15	3.02	2.78
May	3.67	3.81	3.43	3.05	2.48
Jun	4.2	4.16	3.68	3.36	2.32
Jul	4.72	4.5	3.93	3.66	3.1
Aug	4.46	4.49	4.09	4.76	3.76
Sep	3.66	3.46	3.56	3.48	3.02
Oct	3.29	3.14	2.32	3.06	2.52
Nov	3.5	3.53	3.13	3.46	1.64
Dec	3.49	3.45	3.08	2.78	2.63
99 Jan	3.28	3.2	3.13	2.68	3.34
Feb	3.19	2.93	2.17	2.36	2.05
Mar	2.07	2.56	1.18		

Table 5. **Diurnal variation in dissolved oxygen (ml/l) in ponds at Chellanam (C_1 & C_2) and Narakkal (N)**

Time	Pond C_1	Pond C_2	Pond N
6:00 AM	1.67	0.5	0.82
9:00 AM	2.4	2.8	3
12 Noon	5.8	3.4	5.65
3:00 PM	6.42	3.8	7.2
6:00 PM	6.9	4.2	9.34
9:00 PM	5.32	3.5	7.89
12 Night	4.01	2.72	4.2
3:00 AM	3.84	0.92	2.05
6:00 AM	0.79	0.55	0.96

Fig. 11 Monthly variation in dissolved oxygen in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)

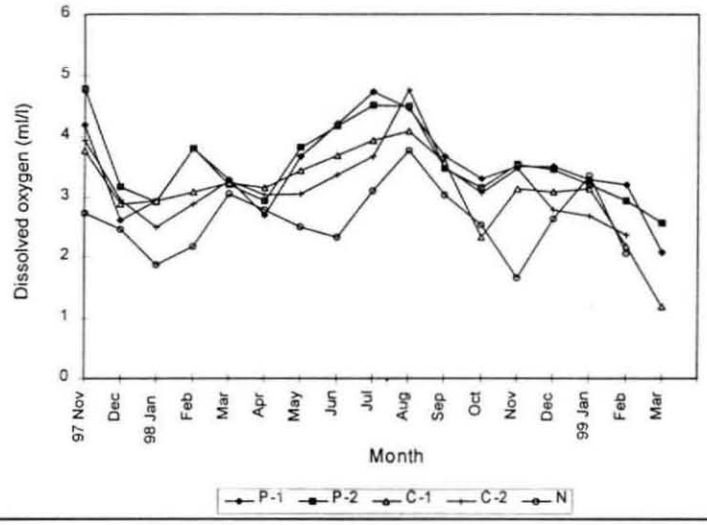
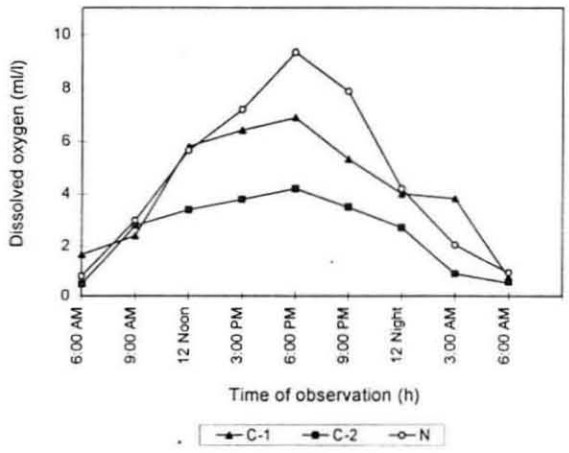


Fig. 12. Diurnal variation in dissolved oxygen in ponds at Chellanam (C_1 & C_2) and Narakkal (N)



sharp rise was recorded in pond N at 06.00 P.M., the values recorded at 06.00 P.M. being, 6.9 ml/l, 4.2 ml/l and 9.34 ml/l respectively in ponds C_1, C_2 and N. Thereafter, a gradual decline in dissolved oxygen was observed in all the three ponds. The minimum dissolved oxygen concentrations of 0.79 ml/l, 0.55 ml/l and 0.96 ml/l were reached in ponds C_1, C_2 and pond N at 06.00 A.M. on the next day (Table 5 and figure 12).

4. WATER pH

In November 1997, the water pH in ponds P_1 and P_2 were 7.49 and 7.53 respectively. Thereafter, a gradual decline in pH values were observed, reaching 7.15 and 7.25 in ponds P_1 and P_2 respectively in January 1998. Again in February 1998, a sharp increase was recorded in both the ponds, the values being 7.72 and 7.76 respectively in ponds P_1 and P_2, followed by a marginal decline till June 1998. Thereafter, a sharp increase was recorded till August 1998. The maximum pH recorded in ponds P_1 and P_2 were 8.17 and 8.15 respectively in July 1998. From September 1998 to March 1999, a marginal decline in water pH was recorded in both the ponds, the pH being 7.22 and 7.17 respectively in ponds P_1 and P_2 in March 1999 (Table 6 and fig. 13).

The recorded water pH in ponds C_1 and C_2 were 7.36 and 7.46 respectively in November 1997. Thereafter, a marginal decline was observed in pond C_1 till June 1998. Whereas, in pond C_2, a gradual decline was observed till March 1998 and after that the pH rose from 7.31 in March 1998 to 7.33 in April 1998. Again the pH fell down to 7.14 in May 1998. A sharp increase in pH was recorded in June and July 1998 in both the ponds. The maximum-recorded water pH was 7.93 in pond C_1 in July 1998 and that in pond C_2 was

Table 6. **Monthly variations in water pH in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	7.49	7.53	7.36	7.46	7.47
Dec	7.21	7.38	7.35	7.47	7.37
98 Jan	7.15	7.25	7.32	7.4	7.33
Feb	7.72	7.76	7.31	7.36	7.15
Mar	7.54	7.58	7.29	7.31	7.03
Apr	7.36	7.4	7.28	7.33	6.91
May	7.34	7.38	7.24	7.14	6.97
Jun	7.76	7.77	7.59	7.66	7.14
Jul	8.17	8.15	7.93	8.17	7.7
Aug	8.15	8.09	7.78	8.18	7.66
Sep	8	8.08	7.38	7.8	7.13
Oct	7.76	7.98	7.41	7.31	7.11
Nov	7.53	7.74	7.35	7.5	7.08
Dec	7.38	7.5	7.57	7.55	7.25
99 Jan	7.38	7.29	7.35	7.4	7.15
Feb	7.15	7.22	7.04	7.2	7.09
Mar	7.22	7.17	7.21		

8.18 in August 1998. Thereafter, a slight fluctuation in water pH was observed in both the ponds, as shown in figure 13.

In pond N, the water pH observed in November 1997 was 7.47 and thereafter, a marginal decline was reported reaching 6.91 in April 1998. From May 1998 onwards, an increasing trend was observed up to August and the maximum value of 7.7 was noted in July 1998. Again pH decreased and a sharp decline (7.13) was recorded in September 1998. The trend of decline persisted till March 1999. The values obtained for water pH in all the five ponds are given in Table 6 and figure 13.

5. NUTRIENTS

a) Ammonia nitrogen

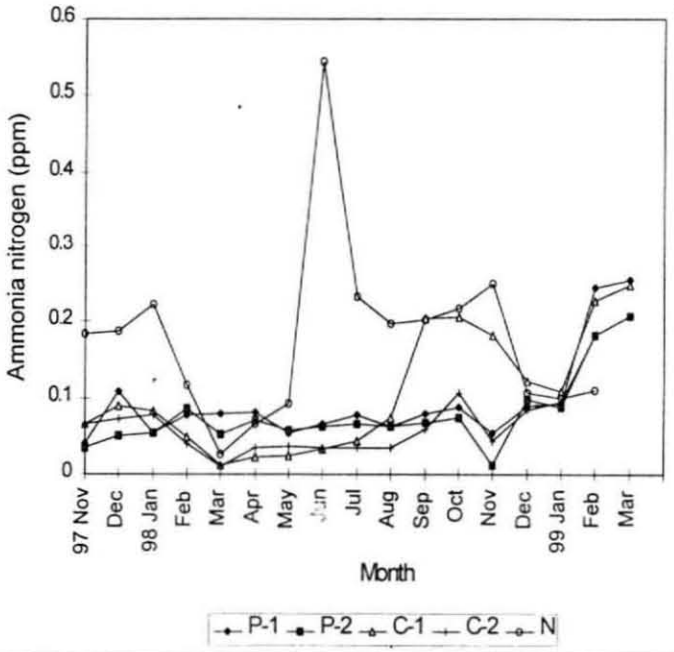
In November 1997, the concentration of ammonia in ponds P_1 and P_2 were 0.0389 ppm and 0.0347 ppm respectively, followed by a marginal increase reaching 0.0808 and 0.0702 ppm respectively in April 1998. In May 1998, a slight reduction was observed. Again the concentration of ammonia increased and remained almost the same with slight fluctuation till October 1998. In November 1998, a sharp decline of 0.0535 and 0.0115 ppm respectively was reported, followed by a sharp increase (0.0885 and 0.0981 ppm in ponds P_1 and P_2 respectively) in December 1998 and this increasing trend continued till March 1999. Maximum concentrations of 0.256 ppm and 0.2082 ppm in ponds P_1 and P_2 respectively were recorded in March 1999 (Table 7 and fig. 14).

The concentration of ammonia recorded in ponds C_1 and C_2 were comparable with that in ponds P_1 and P_2. In November 1997, the reported

Table 7. **Monthly variations in ammonia (ppm) in water recorded in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 &C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	0.0389	0.0347	0.0652	0.0644	0.1836
Dec	0.1083	0.0492	0.0884	0.0721	0.1863
98 Jan	0.0535	0.0527	0.0827	0.0771	0.223
Feb	0.0778	0.0851	0.0475	0.04	0.1159
Mar	0.0792	0.0511	0.0114	0.0103	0.026
Apr	0.0808	0.0702	0.022	0.0349	0.0653
May	0.053	0.0574	0.0235	0.0358	0.0932
Jun	0.0654	0.0612	0.0332	0.035	0.5437
Jul	0.0777	0.065	0.0429	0.034	0.2334
Aug	0.0616	0.0615	0.0742	0.0345	0.1977
Sep	0.0784	0.0673	0.204	0.0591	0.2026
Oct	0.088	0.0729	0.2062	0.1071	0.2171
Nov	0.0535	0.0115	0.1815	0.0425	0.2508
Dec	0.0885	0.0981	0.1209	0.0839	0.107
99 Jan	0.0922	0.0875	0.1086	0.0948	0.0989
Feb	0.2455	0.1821	0.2275	0.1814	0.1097
Mar	0.256	0.2082	0.2484		

Fig. 14. Monthly variation in ammonia in water from ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)



values in ponds C_1 and C_2 were, 0.0652 and 0.0644 ppm respectively, followed by a gradual rise till January 1998. Thereafter, a marginal decline was observed and the minimum concentrations of 0.0114 and 0.0103 ppm were reported in March 1998 in ponds C_1 and C_2 followed by a marginal increase during the next eight months. In November 1998, the concentration of ammonia again decreased to 0.1815 and 0.0425 ppm respectively in ponds C_1 and C_2, followed by a sharp increase till the last month (March 1999) of culture. The maximum concentration of ammonia in pond C_1 was 0.2484 ppm in March 1999 and that in pond C_2 was 0.1814 ppm in February 1999 (Table 7 and fig. 14).

In pond N, the recorded ammonia concentration in November 1997 was 0.1836 ppm which was slightly higher than the previous four ponds. Thereafter, a marginal increase was noted till January 1998, followed by a sharp decline, reaching the minimum concentration of 0.026 ppm in March 1998. Again the concentration of ammonia rose gradually and a very sharp increase of 0.5437 ppm was recorded in June 1998. This was followed by a sharp decline of 0.2334 ppm in July 1998 and thereafter, a gradual increase was noted till November 1998. The values again decreased to 0.107 ppm in December 1998 and started increasing thereafter till the end of the period of study in February 1999 (Fig. 14).

b) Nitrite

In the beginning of culture in November 1997, slightly higher concentration of nitrite (0.0111 ppm) was reported in pond P_1, compared to that in pond P_2 (0.0051). Thereafter, increases of 0.0156 and 0.0105 ppm were noted in ponds P_1 and P_2 respectively in December 1997, followed by a marginal decline in both the ponds till March 1998. The nitrite concentration

suddenly rose to 0.0209 and 0.025 ppm respectively in ponds P_1 and P_2 in April 1998. Again a rise was observed in pond P_1 in May 1998 also and a maximum value of 0.0235 ppm was recorded in this month. Whereas in pond P_2, a marginal decline was observed in May 1998, followed by a sharp decline in both the ponds till August 1998. The minimum nitrite concentration observed in August 1998 in ponds P_1 and P_2 were 0.0013 ppm and 0.0014 ppm respectively. Again a marginal increase was recorded till October 1998, followed by a decline of 0.0023 and 0.002 ppm in ponds P_1 and P_2 respectively and thereafter, almost a steady trend was observed until the end of culture period as shown in table 8 and figure 15.

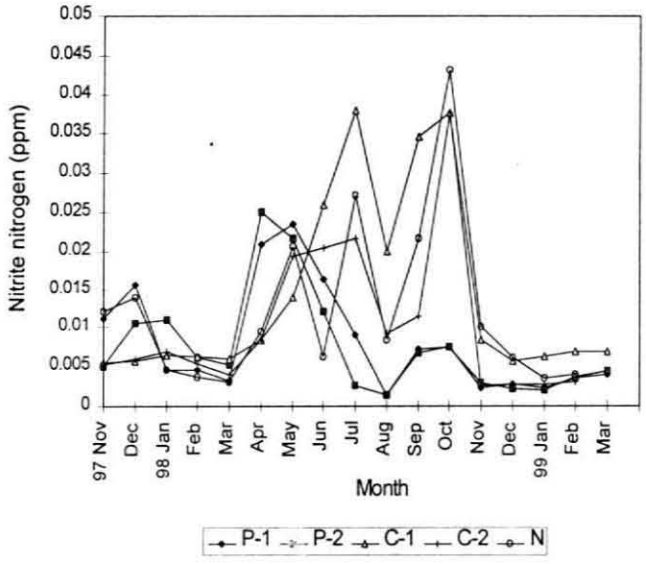
In ponds C_1 and C_2 also, a marginal increase in nitrite concentration was observed from November 1997 to January 1998, followed by a gradual decline till March 1998. A sharp increase was observed in pond C_1 till July 1998, in which month, the maximum recorded value of 0.038 ppm was observed. In pond C_2, a gradual increase was recorded. In August 1998, nitrite concentration in both the ponds showed a sharp decline, followed by a sharp rise till October 1998. Thereafter, sudden decreases of 0.0084 and 0.0026 ppm respectively were observed in ponds C_1 and C_2 in November 1998 and nitrite concentration remained almost stabilised in the following months as seen in figure 15.

The concentration of nitrite recorded in pond N was 0.012 ppm in November 1997. This was followed by a gradual increase in December 1997. The nitrite concentration suddenly decreased from 0.0139 ppm in December 1997 to 0.0046 ppm in January 1998. Thereafter, a gradual decline was recorded till March 1998. But, a sharp increase of 0.0206 ppm in pond N was

Table 8. **Monthly variations in nitrite (ppm) recorded in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	0.0111	0.0051	0.0055	0.0052	0.012
Dec	0.0156	0.0105	0.0057	0.0059	0.0139
98 Jan	0.0045	0.011	0.0064	0.0068	0.0046
Feb	0.0046	0.0061	0.0062	0.0054	0.0037
Mar	0.0032	0.0052	0.0059	0.0039	0.0031
Apr	0.0209	0.025	0.0084	0.0084	0.0094
May	0.0235	0.0216	0.0138	0.0193	0.0206
Jun	0.0163	0.0121	0.0259	0.0205	0.0063
Jul	0.009	0.0026	0.038	0.0216	0.0272
Aug	0.0013	0.0014	0.0199	0.0091	0.0084
Sep	0.0071	0.0067	0.0346	0.0114	0.0216
Oct	0.0075	0.0074	0.0377	0.0376	0.0431
Nov	0.0023	0.0029	0.0084	0.0026	0.0101
Dec	0.0028	0.0021	0.0057	0.0027	0.0061
99 Jan	0.0023	0.002	0.0063	0.0028	0.0035
Feb	0.0035	0.0037	0.0068	0.003	0.004
Mar	0.004	0.0044	0.0069		

Fig. 15. Monthly variation in nitrite in water from ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)



noted in May 1998, followed by sharp fluctuations were recorded till November 1998. The maximum nitrite concentration of 0.0431 ppm in pond N was reported in October 1998. From December 1998 onwards, a marginal decline in nitrite concentration was observed till February 1999 (Table 8 and fig. 15).

c) Nitrate

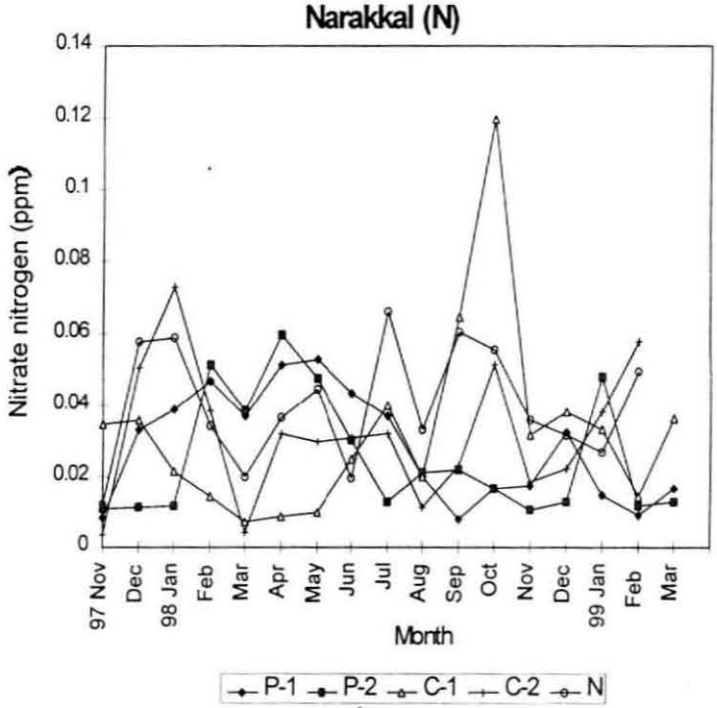
The initial concentration of nitrate recorded in ponds, P_1 and P_2 in November 1997 were 0.0082 ppm and 0.0109 ppm respectively followed by a marginal increase in both the ponds till February 1998. In March 1998, slight decline of 0.0371 and 0.0386 ppm were observed in ponds P_1 and P_2 respectively followed by a trend of increase up to June 1998. Thereafter, nitrate concentration observed in ponds P_1 and P_2 exhibited a fluctuation till the end of culture in March 1999 (Table 9 and fig. 16).

In the beginning of culture in November 1997, the nitrate concentration observed in ponds C_1 and C_2 were, 0.0346 ppm and 0.0034 ppm respectively. In pond C_1, a marginal decline in nitrate concentration was observed till March 1998. This was followed by a gradual rise up to June 1998 and a sharp increase (0.04 ppm) in July 1998. In pond C_2, a sharp increase (0.0727 ppm) was observed in January 1998, followed by a sudden drop in nitrate concentration in March 1998 and the nitrate concentration reported in March 1998 was 0.0043 ppm. Thereafter, a fluctuating trend was observed till the end of culture in March 1999. In pond C_1, the nitrate concentration showed a sharp increase till October 1998, reaching the maximum concentration of 0.1195 ppm in October 1998. Thereafter, the values recorded a fluctuating trend as observed in table 9 and figure 16.

Table 9. **Monthly variations in nitrate (ppm) in water recorded in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	0.0082	0.0109	0.0346	0.0034	0.0121
Dec	0.0333	0.0112	0.036	0.0507	0.0576
98 Jan	0.0389	0.0118	0.0216	0.0727	0.0589
Feb	0.0468	0.0512	0.0144	0.0385	0.0345
Mar	0.0371	0.0386	0.0071	0.0043	0.02
Apr	0.0514	0.0598	0.0086	0.0322	0.0366
May	0.0528	0.0474	0.01	0.0298	0.0444
Jun	0.0434	0.0302	0.025	0.0309	0.0197
Jul	0.037	0.0129	0.04	0.032	0.0659
Aug	0.0199	0.0213	0.0201	0.0114	0.0333
Sep	0.008	0.0219	0.0647	0.0231	0.0604
Oct	0.0166	0.0167	0.1195	0.0513	0.0553
Nov	0.0173	0.0104	0.0317	0.0185	0.0357
Dec	0.0323	0.0129	0.0382	0.0221	0.0317
99 Jan	0.0148	0.0479	0.0331	0.0381	0.0269
Feb	0.0092	0.0117	0.0142	0.0579	0.0493
Mar	0.0166	0.0129	0.0361		

Fig. 16. Monthly variation in nitrate in water from ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)



The nitrate concentration of 0.0121 ppm recorded in pond N in November 1997 showed a sharp increase in December 1997 (0.0576 ppm) and January 1998 (0.0589 ppm) and a decline till March 1998. Thereafter, a fluctuation in nitrate concentration was observed until the last month of culture. The nitrate concentration observed in all the five ponds were given in table 9 and the general trend recorded is shown in figure 16.

d) Phosphate

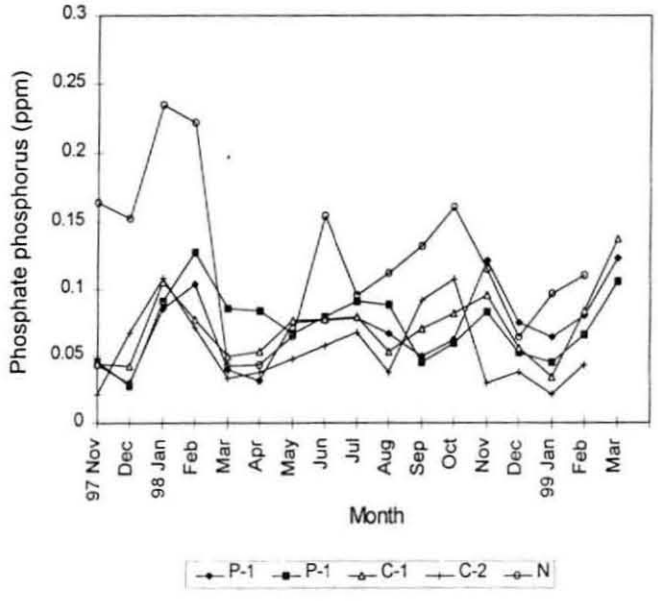
The phosphate concentration recorded in November 1997 in ponds P_1 and P_2 were 0.0427 ppm and 0.0453 ppm respectively, followed by an increasing trend till February 1998 in both the ponds. Thereafter, marginal declines, followed by a marginal rise were observed in the pond P_1 till November 1998. Whereas, in pond P_2, the above trend in phosphate concentration was observed until July 1998, followed by a fluctuation till the end of culture in March 1999. In pond P_1 also, phosphate concentration fluctuated between December 1998 and March 1999, with the maximum concentration being 0.1228 ppm in March 1999 (Table 10 and fig. 17).

The recorded concentration of phosphate in ponds C_1 and C_2 were 0.0433 ppm and 0.0207 ppm respectively in November 1997, followed by a sharp increase till January 1998. Thereafter, a marginal decline was observed in both the ponds up to April 1998, followed by a marginal rise till July 1998. From August 1998 to March 1999, a trend of fluctuation was observed in both the ponds, as shown in table 10 and figure 17. The maximum phosphate concentration of 0.038 ppm and 0.0376 ppm were recorded in ponds C_1 and C_2

Table 10. **Monthly variations in phosphate (ppm) in water recorded in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	0.0427	0.0453	0.0433	0.0207	0.1638
Dec	0.0294	0.0272	0.0414	0.0672	0.1522
98 Jan	0.0855	0.0902	0.1049	0.1083	0.2351
Feb	0.103	0.1268	0.0767	0.0705	0.2224
Mar	0.0388	0.0853	0.0485	0.0327	0.0421
Apr	0.031	0.0834	0.053	0.037	0.0427
May	0.0744	0.0665	0.0763	0.0474	0.0647
Jun	0.076	0.0784	0.0774	0.0573	0.1539
Jul	0.0776	0.0903	0.0784	0.0672	0.0955
Aug	0.0666	0.0879	0.0524	0.0375	0.1112
Sep	0.0485	0.0446	0.0698	0.0918	0.1312
Oct	0.0615	0.0585	0.0815	0.1072	0.1607
Nov	0.1203	0.0828	0.0951	0.0291	0.1138
Dec	0.0744	0.0517	0.0556	0.0369	0.0634
99 Jan	0.0634	0.044	0.0336	0.0207	0.0957
Feb	0.0802	0.0652	0.0834	0.0427	0.1099
Mar	0.1228	0.1047	0.1365		

Fig. 17. Monthly variation in phosphate in water from ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)



during the months of July 1998 and October 1998 respectively (Table 10 and fig. 17).

Comparatively higher phosphate concentration was observed in pond N in the beginning of culture. A phosphate concentration of 0.1638 ppm was recorded in November 1997, followed by a marginal decline (0.1522 ppm) in December 1997 and a sharp increase (0.2351 ppm) in January 1998. Thereafter, a sharp decline was observed in March 1998 and again the graph showed an increasing trend till June 1998. In July 1998, phosphate concentration again decreased, reaching 0.0955 ppm followed by a steady increase (0.1607 ppm) in October 1998. Thereafter, a sharp decline was observed in December 1998 (0.0634 ppm) followed by an increasing trend towards the end of culture in February 1999 (Table 10 and figure 17).

SOIL QUALITY PARAMETERS

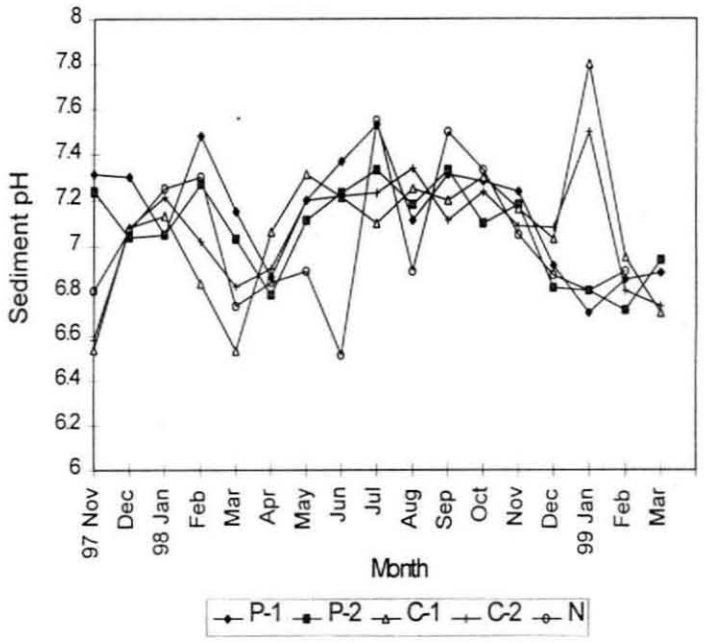
i) Sediment pH

Sediment pH in ponds P_1 and P_2 were comparatively higher and the values recorded were 7.31 and 7.24 respectively in November 1997, followed by a fluctuating trend up to March 1998. In April 1998, both the ponds recorded sediment pH below 7.0 and the corresponding values were 6.86 and 6.78. Thereafter, a sharp increase was observed till July 1998, and the maximum sediment pH of 7.53 and 7.33 were recorded in July 1998 in ponds P_1 and P_2 respectively. Again a fluctuation in sediment pH was observed in both the ponds till December 1998. This was followed by a marginal decline and again a rise (6.88 and 6.94 in ponds P_1 and P_2 respectively) towards the end of the period of study as recorded in table 11 and figure 18.

Table 11. **Monthly variations in sediment pH recorded in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	7.31	7.24	6.54	6.58	6.8
Dec	7.3	7.04	7.08	7.08	7.07
98 Jan	7.05	7.05	7.13	7.21	7.25
Feb	7.48	7.27	6.83	7.02	7.3
Mar	7.15	7.03	6.53	6.82	6.73
Apr	6.86	6.78	7.06	6.9	6.84
May	7.2	7.11	7.31	7.2	6.89
Jun	7.37	7.23	7.21	7.22	6.51
Jul	7.53	7.33	7.1	7.23	7.55
Aug	7.11	7.18	7.25	7.34	6.89
Sep	7.31	7.33	7.2	7.11	7.5
Oct	7.28	7.1	7.3	7.24	7.33
Nov	7.24	7.18	7.16	7.09	7.05
Dec	6.91	6.81	7.03	7.08	6.87
99 Jan	6.7	6.8	7.8	7.5	6.8
Feb	6.85	6.71	6.95	6.8	6.89
Mar	6.88	6.94	6.7	6.73	

Fig. 18. Monthly variation in sediment pH in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)



In the beginning of culture in November 1997, comparatively lower sediment pH were recorded (6.54 and 6.58) in ponds C_1 and C_2, followed by a marginal increase till January 1998. Again in February 1998 and March 1998, there was a sudden drop in sediment pH in both the ponds. Thereafter, the pH values showed a marginal increase till July, followed by a fluctuating trend in both the ponds up to March 1999. The maximum sediment pH were reported in the month of January 1999, the values being 7.8 and 7.5 in ponds C_1 and C_2 respectively (Table 11 and fig. 18).

In pond N, the sediment pH recorded in November 1997 was 6.8. Thereafter, a marginal increase was observed till February 1998, followed by a sudden decline in March 1998. Again a sharp decline was noted in June 1998, which was the minimum sediment pH value (6.51) reported in pond N, followed by a sharp increase in the next month, reaching the maximum pH of 7.55. Thereafter, a marginal decline was recorded in pond N, as shown in table 11 and figure 18.

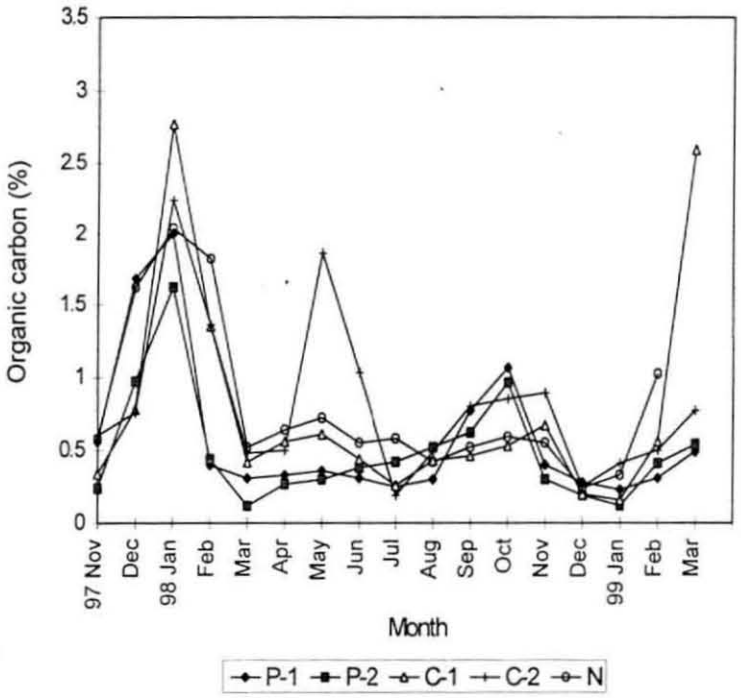
ii) Organic carbon

In November 1997, the organic carbon content in ponds P_1 and P_2 were 0.56 and 0.24 % respectively, followed by a sharp increase up to January 1998. Maximum organic carbon content was reported in ponds P_1 and P_2 in January 1998 and the respective values were 2.0 % and 1.62 %. Thereafter, a declining trend was observed, as shown in fig. 19 till March 1998, followed by a marginal rise up to October 1998 in both the ponds. Again a sharp decline was recorded from November 1998 onwards until January 1999, followed by a marginal increase up to March 1999 (Table 12 and fig. 19).

Table 12. **Monthly variations in organic carbon (%) in sediment recorded in ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 &C_2) and Narakkal (N)**

Months	Pond P_1	Pond P_2	Pond C_1	Pond C_2	Pond N
97 Nov	0.56	0.24	0.34	0.6	0.58
Dec	1.68	0.97	0.78	0.76	1.62
98 Jan	2	1.62	2.77	2.24	2.04
Feb	0.4	0.44	1.35	1.36	1.82
Mar	0.31	0.12	0.42	0.48	0.52
Apr	0.33	0.27	0.56	0.5	0.64
May	0.36	0.3	0.61	1.86	0.72
Jun	0.31	0.38	0.44	1.03	0.55
Jul	0.25	0.42	0.26	0.19	0.58
Aug	0.3	0.52	0.43	0.49	0.42
Sep	0.77	0.62	0.46	0.8	0.52
Oct	1.06	0.96	0.53	0.85	0.59
Nov	0.4	0.3	0.67	0.89	0.55
Dec	0.28	0.19	0.2	0.25	0.24
99 Jan	0.23	0.12	0.16	0.41	0.33
Feb	0.31	0.41	0.56	0.5	1.02
Mar	0.49	0.54	2.59	0.77	

Fig. 19. Monthly variation in organic carbon in sediment from ponds at Pallipuram (P_1 & P_2), Chellanam (C_1 & C_2) and Narakkal (N)



The organic carbon content recorded in ponds C_1 and C_2 were comparable. In November 1997, percentage of organic carbon observed in ponds C_1 and C_2 were, 0.34 and 0.6 % respectively, followed by a sharp increase in December 1997 (0.78 % in each pond) and January 1998 (2.77 % and 2.24 % respectively). Thereafter, a sudden drop in organic carbon was observed till March 1998. In April 1998 and May 1998, again the graph exhibited a marginal increasing trend, which continued up to October 1998. In December 1998, again a sharp decline in organic carbon percentage was observed in both the ponds, the values were 0.2 % and 0.25 % in ponds C_1 and C_2 respectively. This was followed by a marginal increase towards the end of the culture period (Table 12 and fig. 19).

Comparably higher values of organic carbon was observed in pond N and the recorded organic carbon in November 1997 was 0.58 %, followed by a sharp increase up to January 1998. A sudden drop in organic carbon (1.82 %) was recorded in March 1998, followed by a slight fluctuation in organic carbon content till the end of culture as represented in table 12 and figure 19.

B) OBSERVATIONS ON WHITE SPOT SYNDROME (WSS)

Symptoms of white spot syndrome (WSS)

Both *Penaeus indicus* and *P. monodon*, collected from the ponds, affected with white spot disease (WSD), exhibited similar symptoms (Figs. 20 & 22). Almost all size groups were affected by WSD, though smaller prawns were the first to die. The infected prawns stopped feeding, exhibited rapid,

erratic, disoriented swimming movements. They came to edges of the pond and lied on their sides or swam slowly on the water surface and finally sank to the bottom of the pond. They did not exhibit any escape reactions and could be easily caught or attacked by the predators. The prawns were very weak, lethargic and in most of the specimens, dirt and algae accumulated on the gills, appendages and body surface, due to the lack of preening activities. The feedline seemed to be empty. A reddish to pinkish discoloration of the cephalothoracic region was a characteristic feature in the initial stages of infection. In heavily infected specimens, reddish discolouration was observed even on the abdominal segments, uropods, telson and appendages, due to chromatophore expansion (Fig. 22). The growth was retarded. In almost all of the infected prawns, a part of one or both the antennae was cut and the eyes became pale and dull, losing their natural glow. Gill covers extended well below the gills and folded on ventral edge. Black or brown gills became apparent either due to destruction of the gill lamellae and melanin formation or choking with fine silt. Hepatopancreas also became enlarged, and turned pale, yellowish white. The ventral region below the telson also showed a pinkish discolouration. Mouth parts, especially, the maxillipeds became black and started to deteriorate.

In the final or advanced stage, small, obscure white spots of about 0.3 to 3.0 mm size were observed on the underside of the cuticle of the cephalothorax, abdominal segments, appendages, telson and uropods, the concentration being more on the carapace (Fig. 21) and the lateral sides of the fifth and sixth abdominal segments, where they appeared initially and later spread over the whole body. Each spot appeared as a protrusion on the inside surface of the carapace. In some areas of the cuticle, numerous adjacent white

Fig. 20. *Penaeus indicus* affected with white spot syndrome

Fig. 21. Carapace of *Penaeus indicus* showing white spots

Fig. 22. *Penaeus monodon* affected with white spot syndrome

Fig. 23. *Metapenaeus dobsoni* affected with white spot syndrome

Fig. 20

Fig. 21

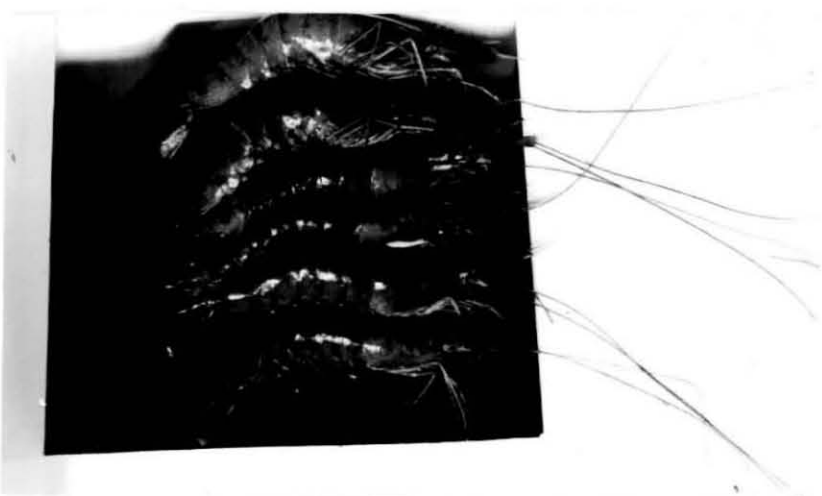


Fig. 22

Fig. 23

spots fused together to form extensive white patches. Another characteristic feature of white spot disease is the loose cuticle. It is easily detachable from the underlying body wall (epidermis). White spots were more clear, when the tissue fragments were scrapped off the detached carapace and observed, by holding it in the sunlight. Loose space was present between cephalothoracic region and the abdomen. In a few specimens, surface of the cuticle was rough, having lost its natural slimy appearance. In spiteof the frequent water exchange, aeration and application of medicines, rapid mass mortality occurred in most cases, within 3 to 5 days, after the symptoms were observed. Dead or moribund specimens were found at the edges and bottom of the pond, in the feed trays and outlet screens. Such dead specimens were in a partly eaten away condition, showing extensive cannibalism. The appearance of white spots on the cuticle was followed by sudden mortality. In the WSD affected ponds at Chellanam, mass mortality of crabs (*Scylla serrata*) and *Metapenaeus dobsoni* was also observed (Fig. 23).

WSD was observed forty days after stocking the grow-out ponds with post larvae in the present study. Eventhough, all the size groups of penaeid prawns were found to be affected with WSD, in the case of *Penaeus indicus*, prawns belonging to the size group ranging from 80 to 120 mm total length and 3.0 to 10.0 g weight in females and 70 to 110 mm total length and 1.0 to 9.0 g weight in males were the mostly affected ones (Figs. 24 & 25). In *P. monodon* also, females of 75 mm to 115 mm total length and 2.0 to 10 g weight and males of 75 to 110 mm total length and 2.0 to 9.0 g weight were the predominant victims of the disease (Figs. 26 & 27). In *Metapenaeus dobsoni*, females of 60 to 80 mm total length and 1.5 to 4.0 g total weight and

Fig. 24. Length-frequency distribution of *Penaeus indicus* affected with white spot disease from ponds at Chellanam

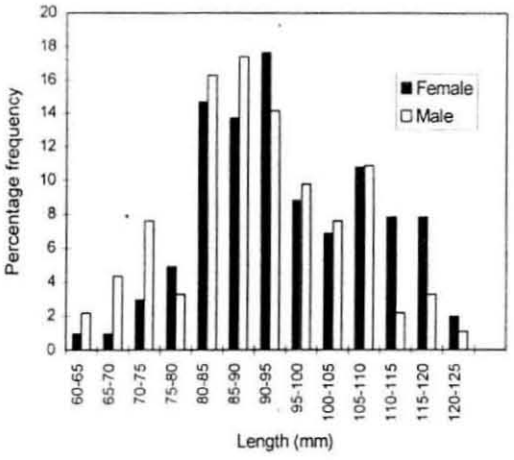


Fig. 25. Size-frequency (weight) distribution of *Penaeus indicus* affected with white spot disease from ponds at Chellanam

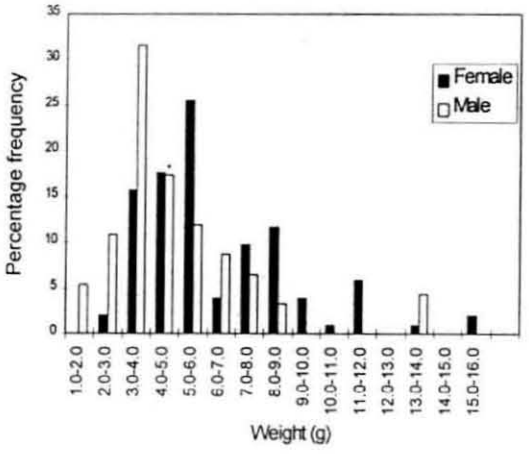


Fig. 26. Length-frequency distribution of *Penaeus monodon* affected with white spot disease from ponds at Chellanam

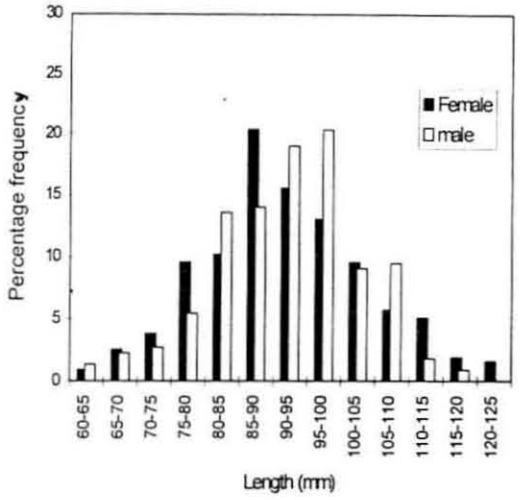


Fig. 27. Size-frequency (weight) distribution of *Penaeus monodon* affected with white spot disease from ponds at Chellanam

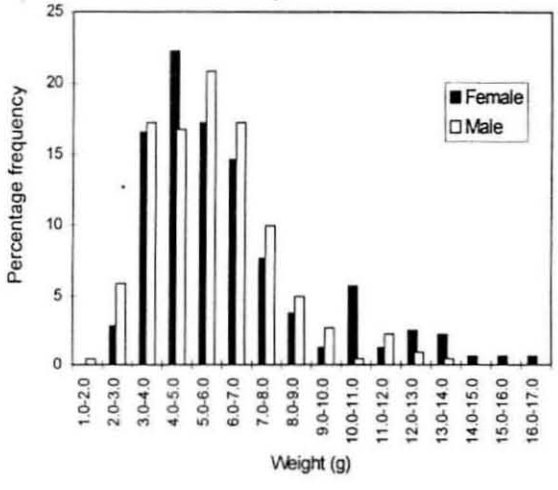


Fig. 28. Length-frequency distribution of *Metapenaeus dobsoni* from white spot disease affected ponds at Chellanam

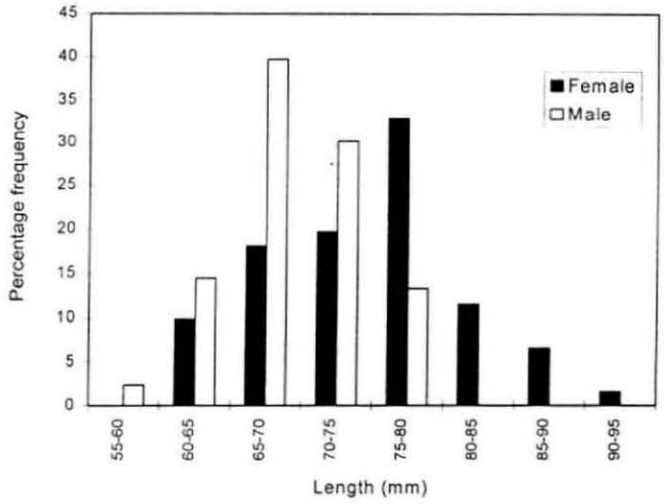
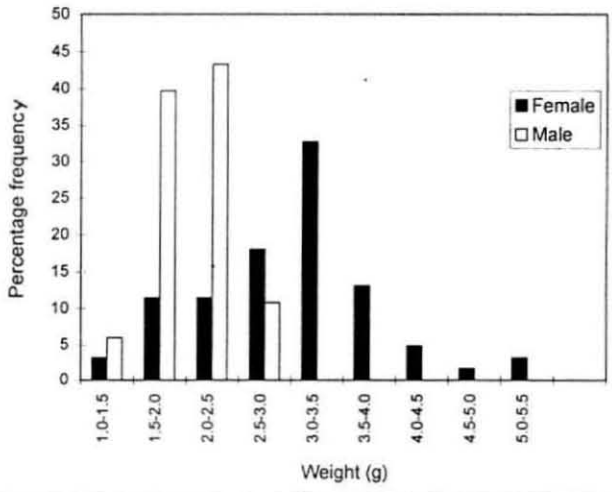


Fig. 29. Size-frequency (weight) distribution of *Metapenaeus dobsoni* from white spot disease affected ponds at Chellanam



males of 60 to 80 mm total length and 1.5 to 3.0 g weight exhibited mass mortality in the present observation (Figs. 28 & 29).

In the experimental study, prawns in tank 1 which were fed with chopped meat from prawns affected with WSD developed symptoms of the disease, such as lethargy, reddish discolouration and appearance of white spots on the third day of feeding and mass mortality occurred on the fifth day. Whereas, those animals in tank 2 exposed to the contaminated water and sediment, developed the symptoms of the disease on the eleventh day of stocking. At fist, two prawns of the stock exhibited severe symptoms and died. Thereafter, the rest of the prawns also stopped feeding, became lethargic and developed white spots on the exoskeleton. Mass mortality was observed on the seventeenth day of stocking. But in tank 3, the animals were in healthy condition and were reared upto thirty days without any ill health.

1.4 DISCUSSION

Fish or prawn under natural condition is in a state of equilibrium with its environment and disease producing organisms, as pointed out by Sneieszko (1973). Any alteration in the environment disturbs this equilibrium, resulting in stress to fish/prawn and they become more vulnerable to disease producing organisms. If the relationship is balanced, good health and growth will occur. If it is marginally changed, it may lead to chronic disease problems and reduced growth. If this relationship is unsatisfactory, poor growth and overt diseases will result. (Das and Das, 1997). Thus, environmental parameters of fish and prawn rearing facilities are of utmost importance for raising a healthy crop.

Environmental parameters include, water quality, soil quality and biological characteristics, of which water quality is of prime importance. In order to maintain water quality parameters within safe limits, one must understand these processes. The factors inhibiting prawn growth and survival should be detected and their impact minimised. In tropical waters, as a general rule, temperature and salinity are higher, metabolic rate, growth rate as well as larval development are faster, thermal tolerance is closer to ambient temperature and lower dissolved oxygen tolerance is closer to ambient levels (Johannes and Betzer, 1975). Although, the primary health problems still relate to biotic agents, in tropical mariculture, fish, shrimp and mollusc display, in general a lower tolerance to the deterioration of physical and chemical qualities of aquatic environment. Sudden fluctuation in temperature, salinity, pH, dissolved oxygen availability, gas pressure, suspended solids etc. can not only render the captive animals more susceptible to disease, but also can be directly responsible for it.

The methods, by which the animals are cultured greatly influence the type and severity of disease. The practices of semi-intensive and intensive culture systems usually result in pollution of the culture medium from uneaten food and waste products of cultured organisms. Therefore, water quality management is one of the most important aspect, especially in semi-intensive and intensive culture systems.

Temperature is considered to be the most important parameter, influencing the various hydrobiological parameters in the culture pond. An increasing trend in temperature towards the summer season months observed in all the five ponds in the present case is a normal phenomenon. The appearance

of white spot disease in ponds, P_1, P_2, C_1 and C_2 were observed in 1998 January, 1998 May, 1998 October and 1999 February. From this, it was very clear that the time of occurrence of the disease in various culture ponds coincided with a slight increase in temperature in all the affected ponds, compared to that of the initial period, the period of stocking. Moreover, the overall temperature range observed during the period when WSD occurred in the ponds was higher than that in the non-occurrence period. So, it could be inferred that the chances of occurrence of WSD might be more during summer months, towards the end of culture period. According to Roberts (1978), pathogenic invasion is likely to occur, at maximum temperature levels.

Though, the temperature range in the present case was within the acceptable level, in the opinion of Roberts (1978), a slight change in temperature, even within this normal range could be a cause of disease. Another factor observed in the present study was that, of all the ponds, maximum temperature (34 °C in 1998 May) was noted in pond N, which was absolutely free from WSD during the entire period of observation. At this time, ponds P_1 and P_2 were affected with WSD. Minimum temperature (27.1 °C in pond C_2) observed during monsoon period was also within the normal range. Slight differences in temperature during a particular culture period in different ponds might be attributed to the different localities. Aquacop (1984) has reported that the optimum temperature for the growth of prawns, *Penaeus indicus* and *P. monodon* are 22 °C to 33 °C and 24 °C to 34 °C respectively. According to Padlan (1990), the desirable temperature range, suitable for the growth of *P. monodon* is 28 °C to 33 °C. In the present case also, the maximum and minimum temperature (34 °C and 27.1 °C) observed in all the culture ponds, during the entire period of study fall within this range. From this

it is very clear that apart from water temperature, some other factors may also be responsible for the occurrence of WSD in the present study.

Diurnal variation in temperature observed in ponds C_1, C_2 and N in the present study also exhibited a diurnal temperature variation as reported by Unnithan (1998) in prawn culture ponds. A minimum temperature of 29.9 °C, 30.8 °C and 30.4 °C were reported in ponds C_1, C_2 and N in the present study at 06.00 AM and maximum values of 35.75 °C, 35.8 °C and 35.6 °C respectively were recorded at 03.00 PM. Unnithan (1998) also reported a minimum temperature of 31.5 °C at 06.00 AM and 35.5 °C at 03.00 PM.

Salinity is the major environmental factor affecting the distribution and physiological processes of organisms living in the brackishwater ecosystems (Kinne, 1966). Salinity of the ponds showed a gradual increase in the summer season. A declining trend was observed during monsoon months. This view is supported by Balakrishnan (1957), George (1958) and George and Kartha (1963), who opined that the salinity of an estuary is that of freshwater during the monsoon and rises near to salinity of seawater during extreme summer months. This statement is true in the case of ponds P_1 and P_2, where salinity rose up to 33.77 and 33.13 ppt respectively during peak summer months. Comparatively higher salinity observed in ponds P_1 and P_2 might be due to the proximity of these regions to the barmouth, when compared to the ponds at Chellanam and Narakkal, which were situated away from the barmouth. WSD was observed in these ponds mostly during summer season, when the salinity was high. Gopinathan *et al.* (1982) also reported higher salinity in ponds at the northern side of Cochin backwaters. Moreover, at the Munambam region, where the ponds P_1 and P_2 were situated, the tidal

influx was also more compared to the other two regions. A decline in salinity during monsoon season was attributed to the heavy rains and river discharges, by which the pond water was diluted. In general, the salinity of the brackishwater pond depends on the salinity of the adjacent estuary.

The salinity range in all the ponds studied was within the normal limit for *Penaeus indicus*. Muthu (1980) stated that a salinity range of 10 to 35 ppt is ideal for the growth of *P. indicus*. The salinity values of ponds P_1, P_2, C_1 and C_2 ponds reached the maximum within the normal range at the time of occurrence of WSD, which might have paved the way for more pathogenic invasion and acted as a slight stress factor to the cultured organisms. Also, salinity at the time of occurrence of WSD was higher in the ponds, than that in the non-occurrence period. The decreased weight and reduced growth of the affected prawns observed in the present study could be attributed to the increase in salinity which might have made the prawns to utilise more energy for osmoregulation.

Dissolved oxygen plays an important role in brackishwater ponds, as a regulator of the metabolic processes of all living organisms, as well as an indicator of the quality of water. Low DO is one of the most common causes of reduced growth and survival of prawns. In the present study, at the time of stocking, the DO content was high in all the ponds. But, as the culture progressed, a trend of declining was observed in the DO of ponds and this might be due to higher rate of organic decomposition and algal growth. However, during the morning hours also, the DO content observed was around 3 ml/l in all the five ponds. At the end of the first culture period, when WSD appeared in ponds P_1 and P_2, the DO concentration was comparatively lower.

In the early culture period also, DO content, slightly lower than 3 ml/l was observed in all the ponds. According to Suseelan (1978), Liao and Murai (1986) and Courtney (1989), the favourable DO concentration for the growth of prawns is above 3 ppm. Kramer (1975), Seidman and Lawrence (1985), Liao and Murai (1986) and Padlan (1990) considered a DO level of 2 ppm to be a stress factor. But in almost all the culture ponds, the chances for DO content to go below the critical levels were high during the early morning hours, due to high rate of organic decomposition and heavy phytoplankton bloom.

Slight differences in the DO in different ponds might be on account of the mode of exchange of water which was exclusively by pumping, resulting in thorough agitation in ponds at Chellanam and Narakkal, in contrast to the water exchange both by pumping and tidal exchange in ponds at Pallipuram. A sharp increase in DO concentration observed during monsoon season was due to heavy rains and high ingress of estuarine water into the ponds. No culture activities were going on during this time. As a whole, the DO content during the period when WSD occurred was lower in the ponds, compared to that in the non-occurrence period of the disease. This might be due to high temperature, more algal growth, and death and decay of plankton and affected cultured organisms.

There was a marked fluctuation in the dissolved oxygen concentration observed during a 24 hour period in the ponds. As in most culture ponds (Boyd and Pillai, 1984), concentration of dissolved oxygen were lowest (1.67, 0.5 and 0.82 ml/l) in the early morning at 06.00 am and increased during day light hours reaching a maximum (6.9, 4.2 and 9.34 ml/l) in the late afternoon at 06.00 pm and decreased again during night in ponds C_1, C_2 and

pond N. Similar trend was reported by Unnithan (1985) in ponds at Vypeen Island. During night hours, since there was only consumption of oxygen by both phytoplankton and algae, minimum DO concentration was observed in the early morning hours. In ponds with extremely dense plankton blooms, it might have gone even below the critical DO levels. Mortality occurs if this critical DO condition continues for a prolonged period. Once the prawns are infected, this critical DO condition may act as a stress factor for them.

The pH in a water body can be influenced by various factors, such as, alkalinity, salinity, temperature and organic matter. During the period of pond preparation and at the time of stocking, the pH of the water was higher due to the application of lime. But, as the culture activities advanced, a slight reduction in pH was observed. This might be due to the combined action of several factors. According to Boyd (1982) and Wickins (1983), a reduction in pH occurred when respiration exceeded photosynthesis and due to nitrification. Moreover, water samples were collected in the morning hours, when the rate of respiration was more and photosynthetic activity was about to begin. Also, there was a slight variation in the water pH of different ponds, probably due to the different ecological locations. A pH range of 7.5 to 8.5 was reported to be ideal for the farming of penaeid prawns (Muthu, 1980). The water pH of all the five ponds examined were above 7.0 and it never exceeded 8.0. This might be due to the type of soil present in the ponds, which showed a lower pH range, as is generally remarked that the pH of water is a manifestation of soil pH (Unnithan, 1985). Eventhough, the ponds at Pallipuram were nearer to the sea, the expected high pH was not recorded, probably because of the leaching effect of the organic residues, an observation made also by Gopinathan *et al.* (1982) and Gopalan *et al.* (1983) in the nearby areas. Boyd (1992) made similar report.

In the present case, the pH values at the time of occurrence of WSD were lower than that during the non-occurrence period. Webber and Webber (1978) and Ferguson (1988) reported poor growth and damage of gill tissue at lower pH.

Ammonia is considered as a common toxicant in aquatic systems and is the principal end product of protein metabolism in crustaceans. Ammonia accounts for 40% to 90% of nitrogenous waste (Hartenstein, 1970; Kinne, 1976; Claybrook, 1983). Safer concentrations of total ammonia of 3.51, 3.7, 3.2 and 4.03 ppm were reported for the growth of juveniles of *Penaeus japonicus* by Chen *et al.* (1990), Chen and Lei (1990) and Kuo and Chen (1991) respectively. In the present investigation, the average concentration of total ammonia observed in the ponds at Pallipuram, Chellanam and Narakkal was within the range of 0.0196 and 0.2875 ppm , which was within the safer limit required for the growth of prawns.

Comparatively higher concentration of ammonia in the range of 0.026 to 0.5437 ppm observed in the pond N in the present study might be either because of low phytoplankton concentration with lesser utilisation of ammonia as a source of nitrogen (Tsai, 1989), or because of feeding the prawns with excess clam meat etc. which might have released ammonia during the process of degradation. In the present investigation, the concentration of ammonia in the culture ponds was low during the occurrence period of WSD, compared to that during the non-occurrence period. Moreover, pond N, which was not affected by WSD during the period of study, had more ammonia concentration when compared to the other ponds, which were affected by WSD. Chin and Chen (1987) also suggested that elevated concentration of ammonia did not initiate lethal damage or the development of white spot syndrome in the

giant tiger shrimp. Contradictory to this, Wang *et al.* (1997b) reported that cumulative mortality of experimental shrimps in rearing water with 2 ppm ammonia was 40% within 14 days of exposure to virus. But control group did not exhibit any disease symptoms or mortality. According to the authors, ammonia had some triggering action in developing white spot syndrome in prawns already infected with the virus. This area needs further experimental studies.

Gopinathan *et al.* (1982) reported that the nitrate concentration in brackishwater ponds near to Cochin estuarine system ranged between 0.003 and 0.069 ppm, with moderate or high values in the middle region and low values at both southern and northern regions of the estuary. Similar observation was made by Gopalakrishnan *et al.* (1988) also, who reported higher nitrate concentration (0.038 to 0.160 ppm) in the ponds at the middle region and low concentration (0.005 to 0.062 ppm) in ponds at the northern end of Cochin estuarine system. Devapiriyan (1990) and Mathews (1992) have also observed higher concentration of nitrates (0.067 to 0.186 ppm and 0.002 to 0.032 ppm) in the brackishwater fields at Vypeen Island situated in the middle region of the estuary. In the present study also, higher concentration of nitrate was observed in ponds at Chellanam (0.0071 to 0.1195 ppm) and comparatively lower concentration was recorded in ponds at Pallipuram and Narakkal (0.008 to 0.0598 ppm and 0.0121 to 0.0659 ppm respectively), which were located in the northern region of the Cochin estuarine system. Variation in nitrate concentration in different ponds at Pallipuram, Chellanam and Narakkal might be on account of the biological utilisation (Upadhyay, 1988) and regeneration from bottom sediment (Mollah *et al.*, 1979).

While investigating the ecological parameters of brackishwater ponds near to Cochin estuarine system, Gopinathan *et al.* (1982) recorded a nitrite concentration of 0.003 to 0.015 ppm. The average nitrite concentration of 0.0013 to 0.025 ppm in ponds P_1 and P_2 at Pallipuram, 0.0026 to 0.0377 ppm in ponds C_1 and C_2 at Chellanam and 0.0031 to 0.0431 ppm in pond N at Narakkal in the present study were slightly higher, compared to the concentration obtained by the above authors. The difference in nitrite values in these two cases might be due to variation in locality, sampling time, season and addition of feed, fertilisers and manure (Boyd, 1982). An upper limit of 0.180 ppm nitrite nitrogen was recommended as the safe level for the growth of *Penaeus indicus* (Jayasankar and Muthu, 1983a). On other hand, Law (1988) reported a high nitrite concentration of 1.28 ppm as the safe level for the growth of *P. monodon*. The concentration of nitrite obtained in the present study was well below the critical concentration with minimum effect on the growth of prawns.

According to Seymour (1980), the high natural level of nutrients, especially phosphorus is essential for activating trophic chains. Orthophosphate is a limiting factor in aquatic systems, influencing the algal growth (Heath *et al.*, 1980). In brackishwater ponds near to Cochin backwaters, Gopalakrishnan *et al.* (1988), Devapiriyan (1990) and Mathews (1992) reported phosphate concentration of 0.005 to 0.160 ppm, 0.042 to 0.159 ppm and 0.004 to 0.144 ppm respectively. In comparison to their observation, the phosphate concentration of 0.0272 to 0.1263 ppm and 0.0207 to 0.1365 ppm recorded in ponds at Pallipuram and Chellanam respectively in the present study were less. This might be on account of the variation in the utilisation of the nutrient (Nair *et al.*, 1988; Upadhyay, 1988). However, in pond N at Narakkal, slightly

higher concentration (0.0421 to 0.2351 ppm) was observed, compared to the above values. A minimum of 0.2 ppm phosphate was considered to be the concentration required for high aquatic productivity (Eren *et al.*, 1977). There was no report regarding the role of phosphate in activating any viral pathogens in the aquatic system.

pH is the most important soil quality parameter influencing the release of nutrients to water body and the rate of decomposition of organic matter. Gopinathan *et al.* (1982) reported a wide range soil pH of 3.5 to 7.0 with low values recorded at the southern and northern parts and higher values in the middle region of the Cochin estuarine system. In the present investigation, the ponds C_1 and C_2 at Chellanam (located in the middle region of Cochin estuarine system) recorded a higher pH range (6.53 to 7.8). Whereas, the ponds at Pallipuram (P_1 and P_2) and Narakkal (N), situated towards the northern side of Cochin estuarine system, recorded a comparatively lower pH range (6.51 to 7.55). These values were in agreement with the observations made by Gopinathan *et al.* (1982). Aquatic weed deposits characterised the areas with low pH in the northern regions of Cochin estuarine system also. But sediment pH range observed in the present case in all the culture ponds examined was within the normal limit of 6.5 to 7.5 (Das and Das, 1997).

According to Gopinathan *et al.* (1982), Ramesan (1990) and Mathews (1992), the organic carbon content in some of the ponds adjacent to Cochin estuarine system, ranged between 0.5% and 4.5%, 0.39% and 2.92%, and 2.41% and 3.79% respectively. On the other hand, Eswaraprasad (1982) and Nasser (1986) recorded comparatively higher values of 1.12% to 6.48% and 2.47% to 8.85% respectively in some of the ponds of the same area. In the

present study, the organic carbon content range reported in ponds at Pallipuram (0.12% to 2.0%), Chellanam (0.16% to 2.77%) and Narakkal (0.24% to 2.04%) respectively were within the normal limit and comparatively lower than those reported by Eswaraprasad (1982), Gopinathan *et al.* (1982), Nasser (1986) and Mathews (1992).

In conclusion, the important water quality and soil quality parameters observed in ponds P_1 and P_2 at the time of occurrence of WSD during the three continuous crops in January 1998, May 1998 and October 1998 respectively were, water temperature- 30.93 °C and 30.4 °C, 33.1 °C and 33.0 °C and 30.0 °C and 30.35 °C, salinity- 26.51 ppt and 26.67 ppt, 31.23 and 30.83 ppt and 4.19 and 4.11 ppt, dissolved oxygen- 2.93 and 2.91 ml/l, 3.67 and 3.81 ml/l and 3.29 and 3.14 ml/l, water pH- 7.15 and 7.25, 7.34 and 7.38 and 7.76 and 7.98, ammonia- 0.0535 and 0.0527 ppm, 0.053 and 0.0574 ppm and 0.088 and 0.0729 ppm, nitrite- 0.0045 and 0.011 ppm, 0.0235 and 0.0216 ppm, and 0.0075 and 0.0074 ppm, sediment pH- 7.05 and 7.05, 7.2 and 7.11 and 7.28 and 7.1 and organic carbon content- 2.0 and 1.62 %, 0.36 and 0.3 % and 1.06 and 0.98 % respectively. In ponds C_1 and C_2, WSD occurred towards the end of February 1999. During this period, the important water quality and soil quality parameters observed were, water temperature- 28.4 °C and 29.4 °C, salinily- 11.1 and 10.3 ppt, dissolved oxygen- 2.17 and 2.36 ml/l, water pH- 7.04 and 7.2, ammonia- 0.2275 and 0.1814 ppm, nitrite- 0.0068 and 0.003 ppm, sediment pH- 6.95 and 6.8 and organic carbon- 0.56 and 0.5 % respectively. All these were within their respective normal range. So, some other extraneous factors along with a combined action of these parameters might be contributing to the occurrence of WSD in the culture ponds in the present investigation.

In the present study, both *Penaeus indicus* and *P. monodon* from ponds, P_1, P_2, C_1 and C_2 were found to be severely affected by WSD. In ponds C_1 and C_2, *Metapenaeus dobsoni* and *Scylla serrata* also exhibited extensive mortality along with affected *P. indicus* and *P. monodon*. Natural infections by WSSV have been reported in *P. chinensis* (= *orientalis*), *P. japonicus*, *P. monodon*, *P. indicus*, *P. merguiensis*, *P. penicillatus* and *M. ensis* (Wang *et al.*, 1995; Durand *et al.*, 1996; Lo and Kou, 1998). In addition, the susceptibility of five species of shrimps (*P. monodon*, *P. indicus*, *P. semisulcatus*, *M. monoceros* and *M. dobsoni*), freshwater prawn, *Macrobrachium rosenbergii*, four species of crabs (*Scylla serrata*, *S. tranquebarica*, *Metapograpsus sp.* and *Sesarma sp.*) and three species of lobsters (*Panulirus homarus*, *P. ornatus* and *P. polyphagus*) from India to WSSV was investigated by Rajendran *et al.* (1999). The disease was experimentally produced in *P.vannamei*, *P. stylirostris* and *P. setiferus* (Lightner, 1996). Lightner *et al.* (1996) recorded natural WSSV infection in *S. serrata* by PCR analysis. PRDV also has a wide host range in decapod crustaceans, including shore crabs, wild prawns etc. (Maeda *et al.*, 1998a).

The principal clinical signs of WSS, such as presence of white spots or patches on the inner surface of the exoskeleton or cuticle of the diseased prawns reported in the present case were similar to that described by Takahashi *et al.* (1994), Anon (1995), Chou *et al.* (1995), Jie *et al.* (1995), Kasornchandra *et al.*, (1998), Wongteerasupaya *et al.*, (1995a), Durand *et al.*, (1996, 1997), Wang *et al.*, (1997b) and Lo and Kou (1998). Penaeid rod-shaped DNA virus (PRDV) infected shrimp also exhibited white spots inside the carapace and reddish discolouration of the body (Momoyama *et al.*, 1994; Takahashi *et al.*, 1994, 1998; Maeda *et al.*, 1998a), as noted in the affected

Penaeus indicus and *P. monodon* in the present study. The dead specimens of *Metapenaeus dobsoni* and *Scylla serrata* from the WSD affected ponds, C_1 and C_2 did not exhibit any gross signs of the disease. Similar observations were reported by Lo *et al.* (1996a) and Peng *et al.* (1998a) in *S. serrata* and according to them, the gross signs of WSS in portunid crabs took the unusual form of clouded areas in the last two segments of the fifth pereiopod. Wang *et al.* (1997a) also did not observe any white spots in the carapace of the infected *M. ensis*. In diseased *P. penicillatus* and *Macrobrachium rosenbergii*, usually white spots could be seen only after the carapace was removed (Peng *et al.*, 1998a). In the present case also, white spots were more clear in the carapace of penaeid prawns, when it was detached and observed in sunlight. Wang *et al.* (1997b) also examined the morphology and composition of white spots on the inside surface of the carapace and opined that white spots were derived from the abnormalities of cuticular epidermis. This might be the reason for the loosening of carapace as noticed in the present case and its easy detachment from the underlying epidermis.

Other signs of WSS, such as reduction in food consumption, lethargy, sluggish movements, reddish to pinkish discolouration and gathering of affected prawns around the edges of the pond and mass mortality, observed in the present study were already reported by Durand *et al.* (1996) and Lo and Kou (1998). In gill-associated virus disease of prawns from Queensland also, Munday and Owens (1998) reported similar lethargy, anorexia and pink to red coloration of appendages and body surface. Prawns affected with yellow head disease also exhibited similar symptoms as reported by Shankar and Mohan (1998). Reduced growth rate and abnormal movements were common features

of many viral diseases, including HPV (Lightner, 1985), MBV (Baticados *et al.*, 1991) and IHHNV (Lightner, 1993).

There was great variation in the extent of white spot infection and the time of appearance of white spots in different species of penaeid prawns. In the present study, the carapace of *Penaeus monodon* exhibited large white patches, compared to small white spots in the exoskeleton of *P. indicus*. In the opinion of Wang *et al.* (1998a), one factor that might be responsible for this was the thickness of the exoskeleton. According to them, *Procambarus clarkii* had a thicker exoskeleton than the prawns and white spots appeared later. On the other hand, the results of *in situ* hybridisation in experimentally infected *P. monodon* (Chang *et al.*, 1996), showed that the cuticular epidermis was initially infected by WSBV at 16h pi and that the degree of infection became serious after 40h pi. This implied that the appearance of white spot syndrome was related to the degree of infection in the cuticular epidermis under the carapace. Present observation also agreed with this suggestion. Moreover, the absence of white spots in the affected *S. serrata* and *M. dobsoni* might have been due to the low degree of WSSV infection in the cuticular epithelium. In cases, where the virus did not affect the epithelium, appearance of white spots could not be used for diagnosis. In severe infection, white spots appeared as patches on the inner surface of the cuticle (Chang *et al.*, 1998).

An empty feed line observed in the affected specimens mighty be due to the absence of food intake, which could have resulted in stunted growth and reduction in the weight of prawns. As the natural slimy appearance of the surface of carapace was lost, it became very rough and could have easily paved way for the attachment of epicommensal organisms, and other secondary

pathogens. This observation coincided with the reports made by Murali Manohar *et al.* (1996) and Vijayan *et al.* (1995), who reported presence of the epicommensal, *Zoothamnium sp.* in *P. monodon*, affected with SEMBV and MBV. Black or brown gills in the affected prawns were a usual phenomenon and a similar case was reported by Prasada Rao (1996). This might be due to destruction of the gill lamellae and accumulation of melanin or choking with fine silt. In the present study, the body surface of the affected prawns generally looked dirty, with sticking of soil and mud due to failure of self cleaning efficiency.

Almost all size groups of the penaeid prawns (*P. indicus, P. monodon* and *M. dobsoni*) were affected by WSD in ponds P_1, P_2, C_1 and C_2, about 40 days after stocking the post larvae in the grow out culture ponds. Mortality of *M. dobsoni* and *Scylla serrata* was noted two weeks after the observing the outbreak of WSD in *P. indicus* and *P. monodon*. According to Bell and Lightner (1987), Chou *et al.* (1995) and Kasornchandra *et al.* (1998), WSD occurred in juvenile shrimps of all ages and sizes, however, the most susceptible ages were between one to two months after stocking in the grow out ponds. This statement supported the present observation. Adults were less severely affected, as their immune defence system was well developed, compared to that of juveniles. Besides this, Peng *et al.* (1998a) suggested that the extent of WSD infection was greater in larvae than in PL. A few adult prawns (above 10.0 g average weight) observed to survive for weeks in the affected ponds was a surprising fact in the present case. Pilot studies conducted by Chou *et al.* (1998) had also shown that the larger, market sized shrimp, *P. monodon* (average weight - 10 g) were relatively non-susceptible to water-borne inoculation and even when fed with WSSV-containing tissue, they

typically did not succumb to the disease. Nonetheless, they were infected and under the stressful conditions of high-density culture systems, this WSBV infection could prove fatal. This phenomenon had also been documented in cases involving MBV, IHHNBV and WSBV, where, increased environmental stress (crowding, shipping , stress from temperature and handling) was positively correlated with increased mortality (Couch *et al.*, 1975; Chen *et al.*, 1989a; Cai *et al.*, 1995; Chou *et al.*, 1995).

Once the symptoms of WSD were well established, mass mortality of penaeid prawns occurred in all the affected ponds. This indicated the seriousness and severity of this dreadful disease. Rajendran *et al.* (1999) also reported a cumulative mortality of 100% within 5 to 7 days in shrimps injected with WSSV and 7 to 9 days in those fed with infected tissues. Nakano *et al.* (1994) could induce 100% mortality in WSBV infected shrimps within 3 to 5 days in infection trials. Another related disease, penaeid acute viremia resulted in 60 to 80% total losses in culture ponds, as reported by Inouye *et al.* (1994, 1996) and Takahashi *et al.* (1994, 1996). Recently reported yellow head virus disease also resulted in a cumulative mortality of 100% within 3 to 5 days in *P. monodon* (Direkbusarakom *et al.*, 1998). Shankar and Mohan (1998) reported that mass mortality occurred within 2 to 3 days of infection in *P. monodon* in India.

In the present investigation, pond P_1 and P_2 had a previous history of WSD infection. During the study period of November 1997 to March 1999 also the disease occurred continuously in the above ponds, causing heavy mortality and losses. In the meantime, in ponds C_1 and C_2, the outbreak occurred only once towards the end of the present study and the mortality rate

and losses were comparatively lower. This could be attributed to the heavy infection of the subcuticular epithelium of the hind gut in the affected prawns as evidenced by the presence of highly basophilic intranuclear inclusion bodies in the hypertrophied nuclei of these cells in the advanced stages of infection, as reported in chapter 2. In the case of severe infection, there might be chances for physical disruption of the overlying cuticle and due to disintegration and lysis of the affected epithelial cells, the inclusion bodies might have come out and passed through the faecal matter, causing further horizontal infection. So released viral particles and those from the dead and decayed organisms could survive in the habitat and might have caused further infection, leading to severe mortality of shrimps stocked in those ponds, which had a history of the outbreak of WSD. Whereas, in newly affected ponds, the concentration of viral particles in water might not be sufficient to produce large scale, sudden mortality. Epidemiological analysis conducted by Mohan *et al.* (1997) of white spot syndrome affected areas along the west coast of India over the last two years had also indicated cases similar to the present observation. The authors attributed this to the occurrence of two forms of WSD, namely, acute form in shrimp culture areas with previous history of the disease causing heavy shrimp mortality and a chronic outbreak in new shrimp culture areas with no such previous history of the disease resulting in delayed or no mortality.

WSD is reported to be transmitted by vertical and horizontal means. As an evidence to the chances of vertical transmission of WSSV, Lo *et al.* (1997) detected WSBV replication in the ovaries, testes and spermatophore of captured brooders of *P. monodon*. Mushiake *et al.* (1999) also reported PRDV in the receptaculum seminis and ovary of spawned broodstock of *P. japonicus*. Moreover, in seed production facilities PAV is shown to be

transmitted vertically from brooders to their offspring by Maeda *et al.* (1998a) and Satoh *et al.* (1999). Cannibalism of the affected prawns by healthy ones was a common phenomenon noted in the present case. The results of the experiment conducted in the present study indicated the chances of WSD transmission by horizontal means, which included feeding the prawns with infected prawn meat and by way of contaminated water and sediment, harbouring the viral particles. Chakraborti *et al.* (1999) also reported that water from shrimp culture ponds experiencing WSSV outbreaks proved WSSV-positive by PCR using WSSV PM primers. Also, actively moving prawns fed on the appendages and body parts of dead ones affected with WSD. This might have led to new infection, and this point was supported by Mohan and Shankar (1998) and Rajan *et al.* (2000), who opined that horizontal transmission could take place by way of carrier organisms, from contaminated water, through co-habitation with infected shrimp, feeding on infected shrimp etc. In all the affected ponds, farmers used clam meat as feed for prawns towards the end of first month of stocking the postlarvae in grow out ponds, which was strongly proved as a bad practice by Liao (1985). The probability of WSBV spreading from coastal regions to culture farms in the interior was high, because shrimp farmers always added raw feeds, such as chopped fish, shrimp, clams and crabs, in addition to artificial diets (Liao, 1985). Therefore, it is better to avoid such raw feeds. To limit the spread of WSD, cleaning and decontamination of ponds should be carried out very carefully, removing potential carriers (Wang *et al.*, 1998b).

To sum up, it is clear that the affected prawns exhibited symptoms, such as occurrence of white spots of 0.3 to 3.0 mm size on the inner side of the exoskeleton of cephalothoracic and abdominal regions, sluggish

movements, reddish discolouration and absence of feed intake. Heavy mortality of *P. indicus, P. monodon, M. dobsoni* and *Scylla serrata* was a characteristic feature in the affected ponds and transmission could occur mainly through horizontal means, such as, cannibalism, feed, water etc. Moreover, WSD appeared 40 days after stocking the prawn culture ponds P_1, P_2, C_1 and C_2 with postlarvae of *Penaeus indicus* and *P. monodon* and so the pathogen was found to be active in a particular size group. Similar to the present observation, Withyachumnarnkul (1999) also reported that in an experimental study, WSS PCR-positive postlarvae of *P. monodon* eventually showed gross signs of white spot disease (WSD) at an average of 40 days after stocking. According to them, PCR screening of postlarvae and rejection of WSSV-positive batches before stocking could greatly improve the chances of a successful harvest.

CHAPTER 2

HISTOPATHOLOGICAL OBSERVATIONS

CHAPTER 2

HISTOPATHOLOGICAL OBSERVATIONS

2.1 INTRODUCTION

Among the various "classical" diagnostic methods, employed in penaeid prawn pathology, the most important ones are the observation of gross and clinical signs, with the most commonly applied laboratory tests being direct examination and microscopy, using the light microscope, classical microbiology with isolation and culture of the agent, and routine histology and histochemistry (Bell and Lightner, 1988; Lightner, 1996). In spite of the introduction of a number of modern techniques and instruments, histopathological studies still remain as important diagnostic tool in primary detection of diseases. This is true in the case of crustacean viral diseases also. Intranuclear inclusion bodies in the target cells are consistent and typical enough in location, morphology and staining characteristics to be considered a good diagnostic criterion (Colorni, 1990).

White spot baculovirus (WSBV) was extremely virulent and had a wide host range. The currently well known hosts of WSBV included penaeid prawns such as, *Penaeus japonicus, P. monodon, P. chinensis* and *P. penicillatus* (Cai *et al.*, 1995; Wang *et al.*, 1995). Histopathological studies had demonstrated that WSBV targeted various tissues, originating from the mesoderm and ectoderm. This was as evidenced by the presence of degenerated cells with hypertrophied or vacuolated nuclei in the tissues examined (Momoyama *et al.*, 1994; Chou *et al.*, 1995; Wang *et al.*, 1995; Chang *et al.*, 1996; Durand *et al.*, 1997). Systemic histological lesions due to WSSV infection were characterised by prominent

intranuclear eosinophilic to basophilic inclusion bodies in the cells of subcuticular epidermis (hypodermis) of the appendages such as pleopods and pereiopods, eyestalks, general body, subcuticular epithelium of foregut and hindgut, pillar and epithelial cells of gill lamellae, various connective tissues, antennal gland, lymphoid organ, hematopoietic tissues, gonads, nervous tissues, haemocytes, heart muscles, striated muscles and in a variety of other cell types (Momoyama *et al.*, 1994, 1997; Wongteerasupaya *et al.*, 1995a; Durand *et al.*, 1997; Wang *et al.*, 1997b; Kasornchandra *et al.*, 1998; Lightner *et al.*, 1998; Nunan *et al,*. 1998). Although, hepatopancreas was not a target organ for WSD virus, virus-infected haemocytes were found between the hepatopancreatic lumens (Wang *et al.*, 1997b).

In the WSSV challenge studies conducted by Lightner *et al.* (1998), histological signs of the infection were first observed in samples taken on day 3 in *P. duorarum* and *P. setiferus*, on day 4 in *P. vannamei* and on day 6 in *P. aztecus*. Severe WSSV infection was observed in samples of all the four species taken between days 4 and 7 after initial exposure. Juvenile *P. aztecus* were found to be more resistant than *P. setiferus* and *P. vannamei*.

Wang *et al.* (1999) experimentally challenged *Litopenaeus vannamei* and *Farfantepenaeus duorarum* with WSSV and their histopathology was studied. According to them, the subgastric artery, gonads and haematopoietic tissues were the most affected areas by WSSV in *Fa. duorarum*. Supamattaya *et al.* (1998) observed that WSSV infected krill, *Acetes sp.* and crabs exhibited hypertrophied nuclei of haemocytes, pilaster cells along the gill filaments and in the subcuticular epithelial cells. Similar changes were observed by Rajendran *et al.* (1999) in the gill lamellae of experimentally infected prawns, crabs and lobsters, along with karryorhexis and pyknosis. According to them, the inclusions found in the gut wall

of mud crabs were more prominent, deeply basophilic, larger and greater in number than those in lobsters and prawns.

According to Momoyama *et al.* (1997) and Wang *et al.* (1997b), the most pronounced destruction was observed in the cuticular epidermis and lymphoid organ, whereas the cuticular epidermis in the stomach and integument were the most heavily affected of all tissues examined. Development of intranuclear hypertrophy and other abnormalities observed in the cells of necrotic tissues were different from stage to stage of the viral infection. No occlusion bodies or cytoplasmic inclusion bodies were observed in the necrotic tissues (Wang *et al.*, 1997a, 1997b).

In the mid stages of virus development, cellular pathology included hypertrophied nuclei with eosinophilic inclusions and marginated basophilic chromatin (Wongteerasupaya *et al.*, 1995a), somewhat reminiscent of the Cowdry A inclusions, characteristic for IHHNV (Lightner, 1993). In the later stages of development, the inclusions were larger and their staining reaction changed from distinctly acidophilic to lightly basophilic with haematoxylin and eosin staining (Wongteerasupaya *et al.*, 1995a). In some cases, both the eosinophilic and the basophilic inclusions were seen together and the transition from eosinophilic to basophilic staining character could be well observed (Durand *et al.*, 1996). Towards the end, the nucleus was disintegrated, leaving enlarged vacant areas (Karunasagar *et al.*, 1997; Kasornchandra *et al.*, 1998). Infected cells were Feulgen-positive, indicating the presence of DNA in the hypertrophied nuclei (Wang *et al.*, 1997b). According to Wongteerasupaya *et al.* (1995a), the state of tissue disintegration was so extensive that no distinctive character could be

attributed specifically to systemic ectodermal and mesodermal baculovirus (SEMBV).

According to Momoyama *et al.* (1997), the size of the abnormal nuclei in *Metapenaeus ensis* from Japan, affected with penaeid acute virus (PAV) remarkably differed depending on the origin of the cells and exhibited a size range of 7 μm to 13 μm in the long axis. In PAV infected *Penaeus monodon* and *P. japonicus* from Taiwan, Wang *et al.* (1997b) reported a size range of 10 μm to 15 μm for the affected nuclei. In the abdominal muscle, heart, lymphoid organ, midgut, nervous tissue, hepatopancreas, testes, ovaries and spermatophores of *P. monodon* from Taiwan, infected nulclei were not so obviously hypertrophied and remained close to the normal size, so they were not readily distinguishable. But, they stained homogeneously with hematoxylin-eosin, similarly to clearly hypertrophied nuclei (Lo *et al.*, 1997). Apart from these, Durand *et al.* (1996), Murali Manohar *et al.* (1996) and Wang *et al.* (1997b) noticed MBV occlusion bodies in the hepatopancreatic tubules and a co-occurrence of *Zoothamnium sp.* in the gill epithelium of *P. monodon.*

In spite of the extensive studies carried out on the histopathology of WSSV in various tissues of penaeid prawns, works done on affected *Penaeus indicus* and other penaeid prawns in our area are very scarce. The present study emphasises the histopathology of various tissues such as cuticular epithelium, alimentary canal, heart and compound eyes of *P. indicus, P. monodon* and *Metapenaeus dobsoni* affected with WSD. The results obtained are discussed in this chapter.

2.2 MATERIALS AND METHODS

A) Collection of animals

Specimens of *Penaeus indicus, P. monodon* and *Metapenaeus dobsoni*, ranging in size from 6.6 cm to 15.7 cm, 6.8 cm to 16 cm and 6.6 cm to 7.8 cm (total length) respectively were collected from white spot syndrome (WSS) affected ponds at Pallipuram, Chellanam, Kumbalanghi, Pallithode and Panangad in Kerala for the present study (Fig. 30). For normal penaeid prawn histology, apparently healthy, *P. indicus* specimens of 6 cm to 15.5 cm in total length were collected from the control pond at Narakkal. Their size, colour, general state of the body and appendages/organs and the behavioural pattern were noted.

Prawns in different stages of infection were observed carefully and the gross morphological studies were made using a folding type 10X magnifying lens. The materials for the microscopic examination included smears, squash preparation of tissues, the whole animals and tissues fixed in suitable fixative, and micro-sections of different tissues. About 500 infected specimens were preserved well in ice taken in sterile plastic covers, brought to laboratory and kept in a deep freezer for further observation and experiment. The procedures followed for sample preparation are given below.

B) Smear

A drop of freshly extracted haemolymph, obtained by cutting the uropods or pereiopod was placed on a clean, sterile glass slide. A thin, uniform smear was drawn by using another slide at 45 °C. The smear is air-dried for a few minutes,

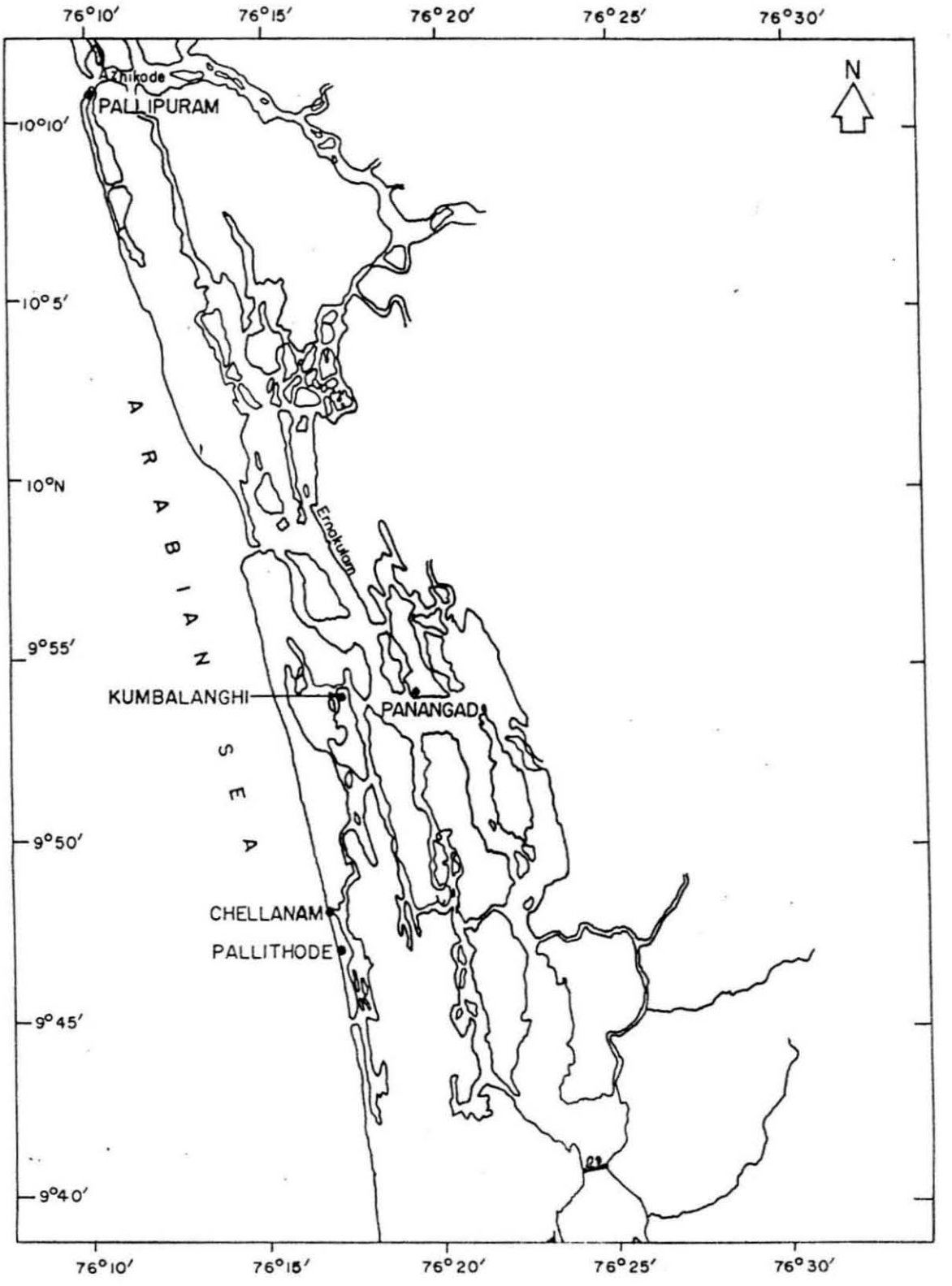


Fig. 30. Map showing areas of specimen collection.

fixed in methanol for five minutes and stained with 0.1% malachite green for 5 to 10 minutes, washed in double distilled water and mounted in DPX.

C) Fixation of materials

Fixation of prawn specimens and the different tissues was carried out at the collection site itself. Davidson's fixative (Bell and Lightner, 1988) was used throughout the study.

The whole prawns were fixed in Davidson's fixative as follows : About 5 ml of the fixative was injected into the prawn, by using a sterile 5 ml syringe and a 21 gauge needle. The sites of injections were, laterally into the hepatopancreas proper, in the region anterior to the hepatopancreas, in the posterior and the anterior abdominal regions, with the hepatopancreatic region receiving the larger volume of the fixative. Soon after injection, the cuticle was slit open from the sixth abdominal segment to the base of the rostrum. Care was taken not to cut deep into the underlying tissues. For larger specimens, two lateral slits were also given, in order to facilitate easy penetration of the fixative. After the incisions, the prawn was fully immersed in the fixative at room temperature. After 48 hours, the properly fixed specimens were transferred to 70% alcohol and stored in separate, labelled bottles.

The organs or tissues, such as, gills, cuticle with the underlying epithelium, foregut, hindgut, compound eye, pleopods, antennae and heart were dissected out from the infected and normal apparently healthy specimens and fixed in Davidson's fixative, the amount of the fixative being at least 10 times the volume of the tissue. Dissections were conducted in crustacean saline for keeping the tissues in the live condition. After 48 hours, all the tissues were directly transferred to 70% alcohol

and stored in separately labelled glass tubes at room temperature, for further processing.

D) Examination of fresh and fixed material in the laboratory

For clinical diagnosis of the disease, the whole body as well as the different organs and cuticle of the fresh and fixed animals were first critically examined with the naked eyes for external symptoms and later, under the dissection or compound microscope. The smear preparations were also examined simultaneously.

Decalcification

Materials, such as, cuticle with the underlying epithelium, antennae, hindgut, compound eye with the stalk were decalcified overnight in 1% nitric acid in 70% ethanol for best results. The decalcified tissues were washed and stored in 70% alcohol.

Processing of tissues and staining

Dehydration and clearing of the tissues were carried out at room temperature as follows :

Properly fixed tissues were dehydrated through, 70%, 80% and 90% alcohol series for one hour each. Two changes were given in absolute alcohol, each for one hour, cleared through a mixture of absolute alcohol and chloroform (1:1 v/v) for one hour and then immersed in pure chloroform for one hour, with two changes for half an hour each. After proper clearing, the tissues become light and translucent.

Then, they were left in a mixture of chloroform and paraffin wax (approximately 1:1) at room temperature, overnight. As a preliminary step to embedding, the tissues were transferred to molten wax (paraffin wax with ceresin, Merck, M.P. 60 °C) for hot impregnation. After three changes of 30 minutes each, tissue blocks were prepared using 'L' blocks and were properly labelled.

Blocks were trimmed well and serial sections of 5 to 6 μ were taken using a manual rotary microtome (Weswox optik, Model MT-1090A). Sections were flattened in a water bath and affixed on clean, glass slides, using fresh Meyer's egg albumin. Subsequently, the slides were dried well by keeping them in a slanting position and staining was carried out with Hematoxylin-Eosin (H & E).

The slides with sections were deparaffinized by giving two changes in xylene and were rehydrated through a descending alcohol series (100%, 95% and 70%). The hydrated slides were stained in Harri's alum hematoxylin (Melby and Norman, 1974) and washed gently in tap water. Excess stain was removed by giving a sudden dip in acid alcohol (1% HCl in 70% ethanol) and the sections were blued in tap water and ammonia water (1% ammonia solution), before counter staining with 1% alcoholic eosin (Pearse, 1972). Eosin stained slides were again dehydrated through 95% and 100% ethanol and cleared in xylene before mounting with DPX. The mounted slides were dried and observed under an Olympus, monocular, compound microscope at varying magnification. Since hypertrophy of the infected nucleus is a prominent characteristic of white spot disease, the micrometry measurements were taken using an ocular micrometer calibrated with a stage micrometer.

E) Squash preparation

Gill filaments from WSS affected animals were fixed for 2 hours, at approximately 25 °C, in Davidson's fixative, which is modified by the replacement of glacial acetic acid with 50% concentrated HCl. They were then rinsed thoroughly with tap water to remove all the acid and stained with H & E as above. While still in xylene, at the end of dehydration, the filaments were dissected into tiny fragments on a clean glass slide, being certain that they were always wet with xylene. Large fragments were removed; a drop of mountant (DPX) was added and the cover slip was pressed to flatten the whole fragments. These were then viewed under the light microscope with high dry lens (20 or 40X).

F) Photomicrography

The stained sections were studied and photomicrographed using 35 mm, ORWO-125, black and white film in Olympus microscope of model, Vanox-AHB-LB, with Koehler illumination and automatic exposure unit.

2.3 RESULTS

The histological changes observed in various tissues such as subcuticular epithelium, gills, alimentary canal, heart and compound eyes of both normal and white spot disease affected prawns are given below.

SUB-CUTICULAR EPITHELIUM

The exoskeleton covering the entire body of penaeid prawns offered protection to the animals and helped in the growth process by moulting. The exoskeleton of control animals was made up of cuticle, which is divided into four layers. The outermost layer was called epicuticle, which lacked chitin, but contained varying levels of calcium. The next layer was the exocuticle, which contained both chitin and calcium. It had melanin like pigments within chromatophores. Next most layer was the endocuticle, containing chitin and calcium. The innermost layer was the membranous or uncalcified layer, containing more of chitin (Fig. 31A).

The body wall of control prawn consisted of an epidermis and a dermis. The cuticular epithelium, immediately following the cuticle was characterised by a single layer of well-organised columnar cells, resting on a basal membrane. Each columnar cell had a basally placed, well defined nucleus, with a centrally located nucleolus. There was artifactual separation of the cuticle from the epidermis. Underlying the epithelial cells was a layer of connective tissue and the component cells, to which the muscles were attached. The connective tissue was fibrous in character and showed a large number of scattered nuclei. Situated within the connective tissue layer were a number of tegumental cuticular glands, each of which consisted of a cluster of secreting cells, with nuclei situated at their bases.

Each gland cells had a capillary canal and the canals of all the gland cells joined together in the centre of the gland to open into a long narrow cuticular duct, leading to the exterior.

In the infected specimens of *Penaeus indicus,* the entire layer of cuticular epithelium, lying just below the cuticle showed the symptoms of white spot disease. The overlying cuticle was seen completely disintegrated and had a granular consistency. Individual layers, such as the epicuticle, exocuticle, endocuticle and the innermost membranous layer were not at all distinct (Fig. 31B). The cuticle was not intact and appeared very loose. The artifactual separation between the cuticle and the epidermis was more in affected specimens than in the control.

Almost all the epidermal cells of severely affected prawns contained markedly hypertrophied nuclei, having highly basophilic intranuclear inclusion bodies in them. In some areas, a cluster of infected nuclei could be seen, indicating hyperplasia. Nucleolus, nuclear membrane and cell boundaries were absent. Basophilic inclusion bodies appeared to be completely filling the hypertrophied nuclei. Multifocal to diffuse necrosis was also observed in some areas. The basophilic inclusions were very prominent and they occupied the entire cell, replacing the cytoplasm in the later stages, and the hypertrophied nuclei of the adjacent cells lied nearby, without leaving much space in between (Fig. 31B). Cellular lesions and tissue disorganisation were clearly evident. Enlarged vacant areas were also seen in some areas, indicating disintegration of the nuclei. In the initial stages of infection, both the eosinophilic and basophilic inclusions were seen together. Some nuclei in the underlying connective tissue of the dermis were also found infected. Haemocytes traversing the dermal layer also exhibited signs

of WSD infection. But, the innermost layer of dermis was found apparently normal. The connective tissue layer also showed large vacant areas in between. The size of the hypertrophied nuclei varied from 4 μ to 14 μ, whereas, nuclei in the control measured 2.6 μ to 6.6 μ in diameter. In the final stages of infection, some basophilic inclusions stained uniformly dark.

The outlying cuticle of *Penaeus monodon*, affected with white spot disease was in a disintegrated condition and the component layers were not distinct. The cuticle was broken in some regions. The entire epithelial layer was infected and the cells had highly hypertrophied nuclei, with basophilic, intranuclear inclusions. In some areas, a few layers of affected nuclei could be observed clustered together and they appeared as deeply stained darker areas. Melanisation and necrosis of such areas were characteristic features. Connective tissues below the epithelial layer was also seen affected, having the characteristic basophilic, intranuclear inclusions in their hypertrophied nuclei. Haemocytes traversing this layer also were infected. But the lower most layer of the dermis was unaffected and had apparently normal nucleus with a clear nucleolus and nuclear membrane. In areas of the epithelium, where large number of hypertrophied nuclei clustered together, marked bulging was noticed. Due to the rupture and disintegration of the epithelial cells, some of the affected nuclei were seen scattered in the cuticular layer also (Fig. 31C). Empty areas were also visible in between. Cytoplasm and cellular boundaries were completely absent. Structural alterations could be observed in the tegumental glands also. The glands become very much compressed and more granular. The size of the hypertrophied nuclei ranged from 5 μ to 14 μ. Some eosinophilic inclusions were also observed.

Fig. 31. Light micrographs showing cuticular epithelium of penaeid prawns

A C.S. of the cephalothoracic subcuticular epithelium of normal *Penaeus indicus* (c_1-epicuticle; c_2-exocuticle; c_3-endocuticle; c_4-membranous layer; ep-epidermis; n-normal nucleus; der-dermis) x 200.

B C.S. of cephalothoracic subcuticular epithelium of *P. indicus* affected with white spot disease (c-cuticle; ep-epidermis; in-hypertrophied infected nucleus with basophilic inclusion bodies; cl-cellular lesion; hem-haemocytes) x 200.

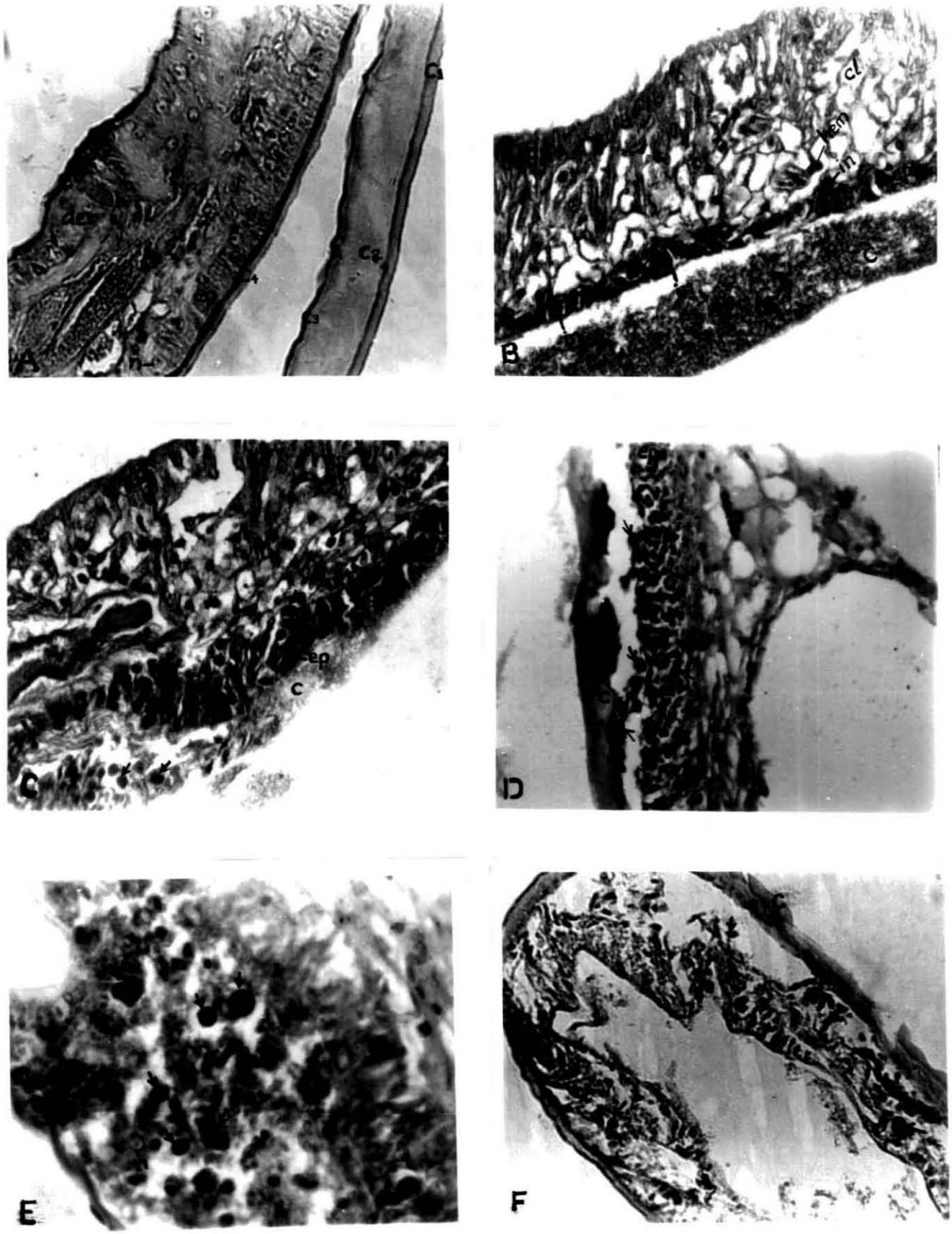
C C.S. of subcuticular layers of *P. monodon* affected with WSD (ep-epidermis, c-cuticle; arrows indicate highly hypertrophied nucleus with basophilic intranuclear inclusion bodies) x 200.

D C.S. of the subcuticular epithelium of antenna of *P. indicus* affected with WSD (c-cuticle; ep-epidermis; arrows indicate hypertrophied nucleus containing basophilic inclusion) x 200.

E C.S. of the subcuticular epithelium of antenna of affected *P. indicus* showing an enlarged view of the infected nuclei (n-normal nucleus; arrows indicate highly hypertrophied nucleus with basophilic inclusion) x 400.

F C.S. of the antenna of *Metapenaeus dobsoni* affected with WSD (c-cuticle; arrows indicate infected nucleus of the subcuticular epithelial cell) x 200.

FIGURE 31



Cuticular epithelium covering the appendages, such as, the antennae, pleopods etc. also showed infected hypertrophied nuclei with eosinophilic to basophilic intranuclear inclusions (Figs. 31D & 31E). In fig. 31D, there existed a wide gap between the antennal cuticle and the underlying epithelium. The inclusion bodies were oval to round in shape. A few apparently normal nuclei could also be seen in between. Several layers of infected nuclei could be observed in the antennal cuticular epithelium in fig. 31D. The cuticle was also severely damaged and broken. Such changes could be observed in the pleopod epithelium of infected *Penaeus indicus*. The affected nuclei were visible in the disintegrated cuticular layer of the antennae, due to the rapture and disintegration of the epidermal cells. Empty spaces or large voids were seen in the epithelial layer and in the cuticle. Some nuclei in the underlying connective tissue also exhibited similar inclusion bodies, but were comparatively smaller when compared to those of the epithelial layer.

Cuticular epithelium of the antennae of *Metapenaeus dobsoni*, collected from the ponds affected with WSD outbreak also showed hypertrophied nuclei with basophilic, intranuclear inclusion bodies. But their size and number were lower, when compared to those of *Penaeus indicus* and *P. monodon*. Melanisation of the affected areas was a noted feature. The size of the hypertrophied nuclei was around 10 μ. Individual cells could not be distinguished and the whole tissue had a granular consistency with basophilic intranuclear inclusions randomly scattered in it (Fig. 31F).

GILLS

In the control, each gill filament had a central axis, which was attached to the cephalothoracic wall, via, a tubular structure, seen at the bottom of

the central axis. The primary filament branched out from the central axis and each primary filament further divided into secondary filaments. The afferent and efferent vessels were seen at the base of the primary filament. A thin septum divided the two adjacent vessels. Secondary gill filaments were of two types- secondary non-branching and secondary branching filaments. Each filament contained basally located afferent and efferent vessels, separated by a thin septum. Outermost layer was the cuticular lining, followed by the gill epithelium. Epithelial pillar cells spanned the distance between the opposite cuticular walls. Each epithelial cell had a well-defined nucleus. The nucleolus was also very clear as seen in figure 32A. The nuclear membrane and cell membranes were quite distinct. With haematoxylin-eosin staining, the nuclear membrane and nucleolus stained deep blue and the cytoplasm stained pinkish. Interspersed between the pillar cells were interconnecting spaces or lacunae, through which the haemolymph moved. Circulating haemocytes, traversing the gill filaments were also visible, which appeared basophilic and spindle shaped. The distal tips of the filaments contained enlarged lacunae, filled with a granular substance.

In the cross section of the gill central axis, the efferent vessel lied dorsal to the afferent vessel. Endothelial cells lined both the vessels. Auxiliary channel was seen juxtaposed between the two vessels. Nephrocytes form a boundary between the efferent vessel and auxiliary channel. Haemocytes were observed within the efferent vessel.

In the affected gill, the outlying cuticle was seen disrupted in some areas of both the secondary branching and non-branching gill filaments (Fig. 32B). Structural disintegration of the entire filament was evident. Masses of affected nuclei could be seen associated with the disintegrated tissue remnants. Infected

haemocytes were also present. In severely damaged gills, the structural alteration was so great that the central axis and the entire tissue appeared as a jelly like mass, traversed with haemocytes and hypertrophied nuclei of the epithelial cells with intranuclear basophilic inclusions. The nuclei of the pillar epithelial cells of both the primary and secondary gill filaments exhibited similar changes, i.e., basophilic intranuclear inclusion bodies and marked hypertrophy. Both eosinophilic and basophilic inclusions were present in the same area. A hyaline, clear area could be discernible in between the eosinophilic and slightly basophilic inclusion bodies and the nuclear membrane. The outer cuticular lining of the gill filaments became very loose and was separated from the underlying epithelial layer in most case. In some secondary filaments, large vacant spaces were seen in between the affected epithelial cells. Individual cells were not visible, as the cell boundaries were lost. Cross-sections of secondary gill filaments were thickly packed with basophilic, hypertrophied nuclei, having intranuclear inclusion bodies. The endothelial cells, lining the afferent and efferent vessels, present in both the primary and secondary gill filaments showed marked hypertrophy, with eosinophilic to basophilic inclusion bodies in them (Fig. 32B). Large voids were also observed in the vessels, replacing the usual granular material. Infected haemocytes were present in the lacunae. Both the afferent and efferent vessels present in the central axis also showed similar changes. Endothelial cells, lining the vessels were the most severely affected group of cells. The size of the hypertrophied nuclei ranged from 3.6 μ to 13 μ in diameter.

Another notable feature was the presence of certain giant cells in the affected gills of *Penaeus indicus*. These cells were mostly round. A few spindle shaped cells were also seen. The nuclei seen in them varied from round to horse shoe in shape. The cytoplasm was eosinophilic to pale basophilic

Fig. 32. Light micrographs of the gill filaments of *Penaeus indicus* and *P. monodon*

A L.S. of a part of gill filament of normal *P. indicus* (p-primary gill filament; s-secondary gill filament; c-cuticular lining; ep-gill epithelium; epc-epithelial pillar cell) x 200.

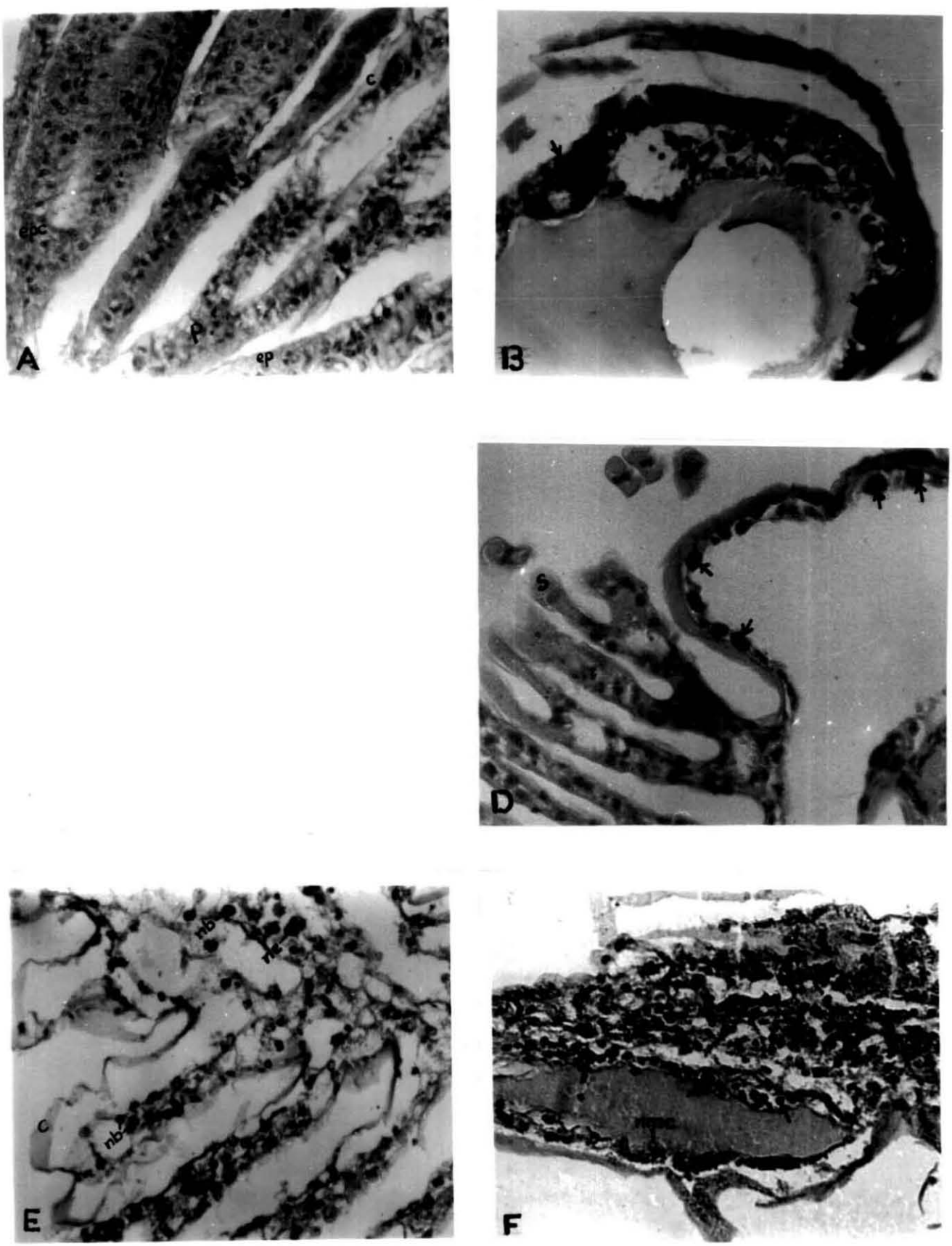
B C.S. of the gill central axis of *P. indicus* affected with WSD (Arrows indicate hypertrophied nuclei with basophilic inclusion bodies in the cells lining blood vessels) x 200.

D C.S. of gill in *P. indicus* affected with WSD (ca-central axis; p-primary gill filament; s-secondary gill filament; arrows indicate affected nuclei of the epithelial cells) x 200.

E C.S. of the gill filament of *P. monodon* affected with WSD (c-cuticle; ne-nucleus containing eosinophilic inclusion; nb-nucleus with basophilic inclusion) x 200.

F C.S. of gill in *P. monodon* affected with WSD (nmc-nucleus showing margination of chromatin; arrows indicate nuclei with basophilic inclusion bodies in them) x 200.

FIGURE 32



Large empty spaces were observed in the central axis also, with the epithelial cells immediately below the cuticle exhibiting symptoms of WSD infection (Fig. 32D).

Lesions, similar to those described above for the WSD affected gills of *Penaeus indicus* were observed in *P. monodon* affected with WSD also. In the gills of *P. monodon*, the outer cuticular lining of the secondary gill filaments were widely separated from the underlying epithelial layer. Both, eosinophilic and basophilic inclusions were observed in the same filament (Figs. 32E & 32F). Margination of the chromatin was visible in some nuclei, as a basophilic ring like or disc like structure in the periphery of the affected hypertrophied nuclei, which contained the eosinophilic inclusions inside. Structural disintegration of the entire gill tissue was a noted feature. Some of the affected nuclei, came out through the broken cuticular lining were observed outside the margin of the secondary gill filaments. A few pillar epithelial cells were found completely lysed leaving large voids in between. The affected nuclei measured 3.3 to 12 μ in diameter.

ALIMENTARY CANAL

Alimentary canal of control prawns consisted of an anterior foregut, hepatopancreas, midgut and posterior hindgut. The foregut and the hindgut had an internal lining of cuticle, continuous with the cuticular covering of the integument, but the long midgut had a soft, endodermal lining. Except for the buccal cavity and the oesophagus, which were placed vertically, the rest of the alimentary canal formed a more or less straight tube, right upto the anus.

a. Stomach

The stomach began anteriorly at the ventral oesophagus and extended posteriorly to the approximate mid point of the hepatopancreas. The stomach was divided into an anterior and posterior chamber; the latter divided again into dorsal and ventral subchambers. Both the regions of the stomach were lined with a layer of cuticle, normally non-calcified, except for individual tooth-like projections, or grinding surfaces (ossicles). The inner wall of the anterior chamber had highly exaggerated folds, projecting into the lumen, where mastication of the consumed food occurred and this region is often known as the "gastric mill". Gastric sieve, which was closely associated with the primary hepatopancreatic ducts, was the major component of the posterior chamber. The gastric sieve was a complex structure of cuticular setae and grooves as seen in fig. 33A.

In the anterior and posterior chamber wall, immediately beneath the thin non-calcified cuticle was the simple, columnar cuticular epithelium, slightly separated from the cuticle. A thick layer of spongy connective tissue, supporting the cuticle and cuticular epithelium was very clearly seen. In the control, (as observed in fig. 33B) individual cuticular epithelial cells were quite distinct, having nuclei with very clear nucleoli. Nuclear membrane and cell boundaries were also clearly seen. The size of the nuclei varied from 3.3 μ to 7 μ. Thick layers of circular and longitudinal muscles surrounded the entire stomach, with spongy connective tissues occupying major regions. Under higher magnification, the sublayers of cuticle, such as, the outermost epicuticle, the underlying exocuticle and the endocuticle were also visible.

Partly masticated food materials were observed in the lumen of the stomach as shown in fig. 33A. In the gastric sieve region, the sieve was seen artifactually separated from its epithelial layer. Longitudinal inter-setal grooves were clearly seen. The tips of the gastric sieve and muscle bundles were seen oriented variously.

The epithelial layer beneath the cuticular lining and the spongy connective tissue in the stomach of WSD affected *Penaeus indicus* was found to be infected by WSV. The nuclei of these cells were highly basophilic, hypertrophied and seemed to occupy the entire cell. They contained intranuclear inclusion bodies, which completely filled the nuclei. The hypertrophied nuclei measured from 6.6 μ to 13.5 μ in diameter. Some hypertrophied nuclei were perfectly round, whereas others, which were heavily infected, showed varying shapes. The basophilic and hypertrophied nature of the infected nuclei, increased in the advanced stages of infection. Empty spaces were observed in between the affected epithelial cells of the posterior stomach (Fig. 33C). In some areas, the cell boundaries were indistinct and so individual epithelial cells were not clearly visible. A group of basophilic, hypertrophied nuclei were seen clustered together as shown in fig. 33C. The spongy connective tissue, lying in between the epithelial layers, appeared to be looser with large voids in between the cells, as compared to those in the control (Fig. 33B). Some of the connective tissue cells also had eosinophilic to slightly basophilic nuclei, without the nucleoli .. In a few, margination of the chromatin near the nuclear membrane was also observed as shown in fig. 33D. Some of the haemocytes were basophilic and hypertrophied. Another characteristic feature was that the stomach cavity was empty, having no remnants of the masticated food materials. Noticeable artefacts were observed in the cuticular lining, immediately adjacent to the infected epithelial layers.

Fig. 33. Light micrographs showing sections of stomach and hepatopancreas in *Penaeus indicus*

A C.S. of the stomach of normal *P. indicus* (vs-ventral subchamber; lsg-longitudinal inter setal groove; ce-cuticular epithelium; sct-spongy connective tissue) x 40.

B An enlarged view of the subcuticular epithelium of the posterior chamber of stomach in normal *P. indicus* (ec-epithelial cell; n-normal nucleus; sct-spongy connective tissue with normal nuclei) x 400.

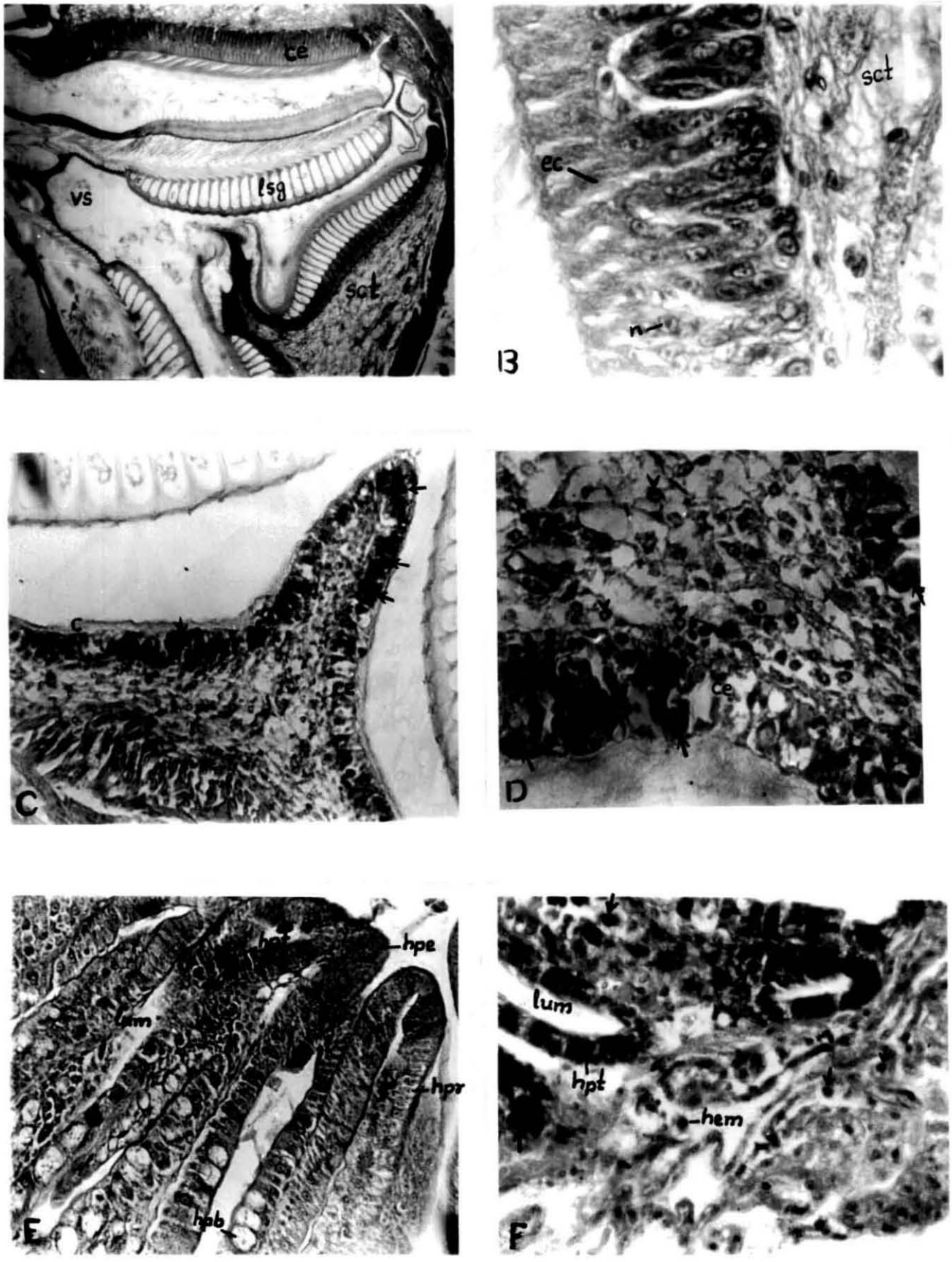
C C.S. of the stomach wall of *P. indicus* affected with WSD (c-cuticle; ce-cuticular epithelium; sct-spongy connective tissue; arrows indicate hypertrophied nuclei with basophilic inclusions in the epithelial cells) x 100.

D An enlarged view of the affected cuticular epithelial cells in the stomach wall of *P. indicus* infected with WSD (ce- cuticular epithelium; arrows indicate affected nuclei of the epithelial cells; arrow heads indicate chromatin margination in the affected nuclei of spongy connective tissue) x 200.

E L.S. of hepatopancreatic tubules in normal *P. indicus* (lum-lumen; hpe-E-cell; hpr- R-cell; hpf- F cell; hpb- B-cell) x 100.

F L.S. of hepatopancreas in *P. indicus* affected with WSD (hpt-hepatopancreatic tubule; lum-lumen; hem-haemocytes with hypertrophied nuclei containing basophilic inclusion; arrows indicate infected haemocytes) x 200.

FIGURE 33



b. Hepatopancreas

The dorsal and ventral lobes of the hepatopancreas surrounded the gastrointestinal tract. In the L.S. of the hepatopancreatic tubule, the transition of the cell types from the new, undifferentiated apical end through the median region and finally to the proximal region was noted (Fig. 33E). The lumen of the tubule contained a granular material and the lumen-facing surface of the cells was covered with a microvillous brush border. The tubule apex contained undifferentiated embryonalzellen cells or E-cells. Mitotic figures and typical binucleated cells were also seen in this region. Proceeding away from the apex, the cells differentiated into absorptive, storage restzellen or R-cells. Another cell type seen in the middle part of the tubule was the fibrillenzellen or F-cells. F-cell nuclei were larger than those of R-cells and had prominent nucleoli. There were numerous vacuoles in the cytoplasm. In addition to the above cell types, the tubule also contained large, distinctive, secretory blasenzellen or B-cells, each of which contained one very large vacuole. The B-cells typically had a convex luminal surface. Myoepithelial cells with prominent nuclei, and associated fibres and haemal sinuses separated adjacent tubules. Haemocytes were also present within the sinuses. At the periphery, a spongy connective tissue sheath surrounded the lobe.

Similar cell types, as described above in the control were seen in the hepatopancreas of the WSD affected *Penaeus indicus* also. The lumen of the tubule was empty, without any secretory granules. Haemal sinuses, present in between the tubules contained haemocytes with hypertrophied and basophilic nuclei, having intranuclear inclusion bodies as seen in fig. 33F. Connective tissue sheath, surrounding the periphery of the lobe also showed basophilic,

hypertrophied nuclei. However, the numbers of affected cells were very low and they were distributed randomly.

c. Midgut

The mucosal epithelial cells were simple, columnar, with median nuclei and prominent nucleoli. The surface adjacent to the lumen had a brush or microvillous border. A thin basement membrane, circular muscle, longitudinal muscle and an outermost layer of fibrous connective tissue or serosa supported the epithelial cells.

In the WSD affected *Penaeus indicus* also, the structure of the midgut was similar to that of the control. The epithelial cells of the midgut had centrally located nuclei with clear nucleoli and nuclear membranes (Fig. 34A). No hypertrophied nuclei with eosinophilic or basophilic inclusions were seen in the midgut epithelial layers. But the posterior aorta or dorsal abdominal artery lying above the midgut in the central midgut region and the subgastic artery showed severe signs of infection and clearly exhibited haemocytes with abnormally hypertrophied nuclei and highly basophilic inclusion bodies in them. These two regions were markedly stained with H& E.

d. Hindgut

Hindgut was seen in the sixth abdominal segment. At the posterior extremity of the midgut lied the dorsally projecting posterior midgut caecum and just posterior to this can be seen the folds of hindgut. The hindgut folds were distally lined with an epithelial layer and composed largely of tegumental glands. The epithelial layers were quite distinct. The anterior ends of the hidgut folds

were covered with bacteria or bacterial flocculent. Beneath this atypical layer, the cuticle lining the hindgut was evident. The cuticle was thin, non-calcified and lied adjacent to the hindgut lumen (Fig. 34B). Underlying the cuticle was the simple (single-cell thick) hindgut epithelia. The nucleus with the nucleolus and nuclear membrane was visible in the epithelial cell. Cell boundaries were also distinct. Occupying the remainder and majority of the hindgut folds were the tegumental glands, which contained large cells with prominent nuclei as seen in fig. 34B. In between the folds of the hindgut, the lumen was visible. The lumen of the hindgut appeared multi-chambered due to extensive folding. Remnants of food materials could be seen in the lumen. The size of the nuclei of the epithelial cells ranged from 3 μ to 7 μ.

Fig. 34C gives an overall picture of the infected hindgut of *Penaeus indicus*. In it, the lumen was almost empty, without the remnants of food materials. In WSD affected *Penaeus indicus*, the nuclei of the epithelial cells, lining the hindgut folds became greatly hypertrophied and contained large basophilic intranuclear inclusion bodies (Fig. 34D). The nucleolus and nuclear membrane were absent and the entire nucleus was filled with the inclusion body. Some eosinophilic inclusions were also seen. But, they were small compared to the basophilic inclusions. In some cases, the entire cell was seen occupied by the hypertrophied nucleus. The size of these infected nuclei ranged from 5 μ to 13.5 μ in diameter. A small 'halo' could be observed around the inclusion bodies, which varied in width. The cellular boundaries were not clear, and the bacterial flocculent at anterior end of the hindgut folds was not seen. The underlying cuticular lining was also not as thick as that in the control. Such inclusions occurred even in the tegumental gland cells. But, their number and size were small, compared to those in the epithelial cells. The shapes of the hypertrophied

nuclei also differed much. Some were oval in shape; most of them attained a round shape. A few elliptical shaped nuclei were also noticed. A small number of apparently normal, epithelial cell nuclei, with nucleoli and well-defined nuclear membranes were also observed in between the affected cells.

The number and sizes of the infected nuclei were larger in WSD affected *Penaeus monodon* than in *P. indicus*. Both eosinophilic and basophilic, intranuclear inclusions of varying shapes and sizes were observed in the hypertrophied nuclei of the epithelial cells, lining the lumen of the hindgut. The cytoplasm immediately surrounding the affected nuclei was disintegrated, leaving voids around them, which appeared as clear areas. Eosinophilic inclusions were seen in the nuclei of the tegument gland cells also. The size of the infected nuclei varied from 4 μ to 14 μ. An empty space could be visible surrounding the hypertrophied nuclei, containing the basophilic inclusions as seen fig. 34E.

Similar observations as seen in *Penaeus indicus* and *P. monodon* hindgut were made in *Metapenaeus dobsoni* also. But in *M. dobsoni*, a portion of the hindgut epithelium was seen severely affected by WSBV (Fig. 34F). In it, a cluster of hypertrophied nuclei, with highly basophilic intranuclear inclusions were seen in one of the hindgut folds. Both the epithelial and tegument gland cells were affected. The nucleolus, nuclear membrane and cell boundaries were completely absent. A few eosinophilic inclusions, with marginally displaced nucleolus were also observed. Individual tegument gland cells were difficult to locate and a marked disintegration of the entire tissue was a characteristic feature of the lesion in this region. Inclusion bodies in different stages of formation and development could be well differentiated in this region. The cuticular lining was not continuous and broken at places. In fig. 34F, fully developed, basophilic

Fig. 34. Light micrographs showing sections of midgut and hindgut in penaeid prawns

A C.S. of midgut from WSD affected *Penaeus indicus* (ep-epithelial cell; hem-haemocyte) x 200.

B C.S. of hindgut in normal *P. indicus* (ep- epithelial layer; lum-lumen; teg-tegumental glands; c-cuticular lining) x 200.

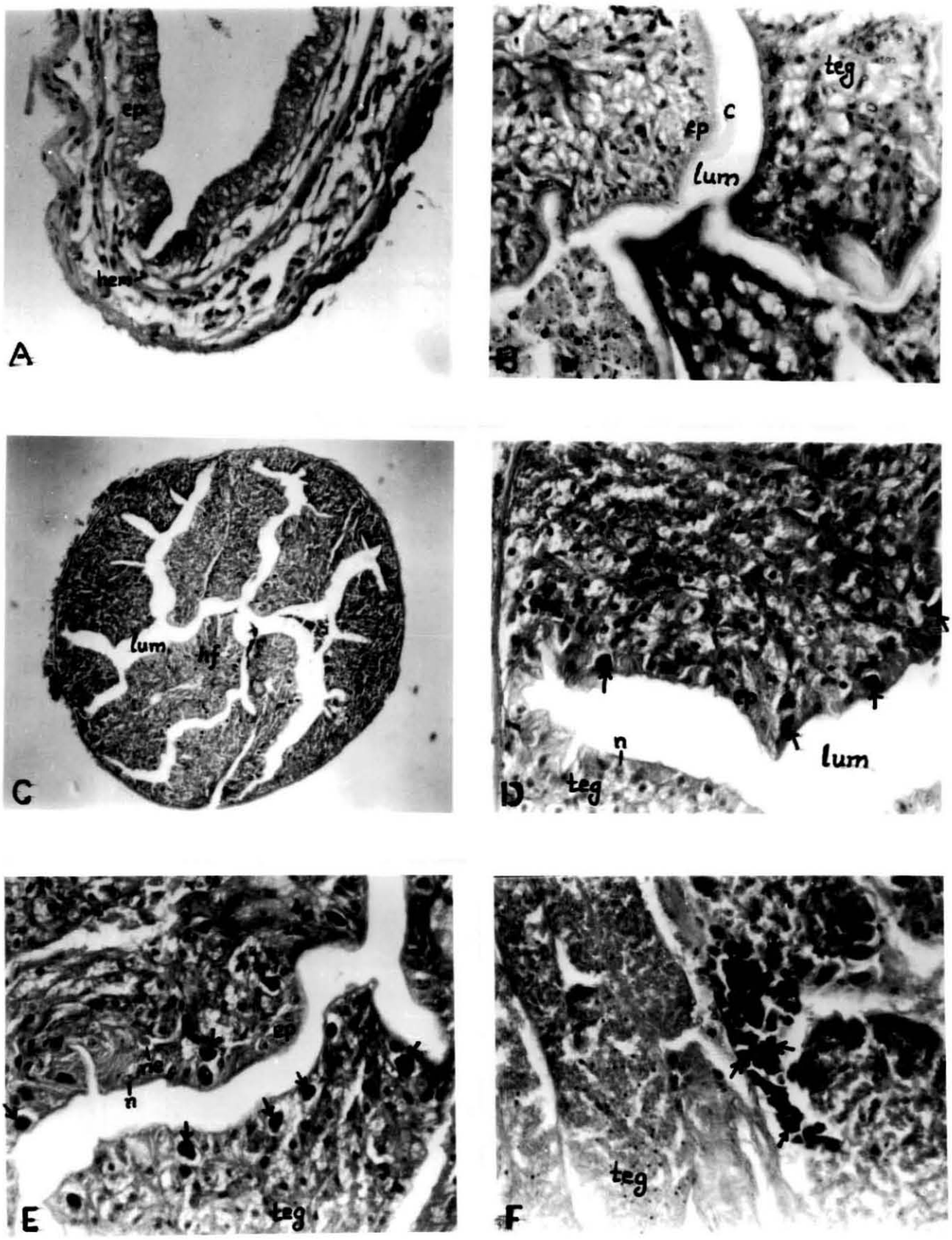
C C.S. of hindgut of *P. indicus* affected with WSD (lum-lumen; hf-hindgut folds) x 40.

D Light micrograph showing enlarged view of hindgut folds of *P. indicus* affected with WSD (ep-epithelium; n-normal nucleus; ne- nucleus having eosinophilic inclusion; teg-tegumental glands; lum-lumen; arrows indicate basophilic inclusions in the hypertrophied nuclei) x 200.

E Enlarged view of hindgut folds of *P. **monodon*** affected with WSD (ep-epithelium; n-normal nucleus; ne- nucleus having eosinophilic inclusion; teg-tegumental glands; lum-lumen; arrows indicate basophilic inclusions in the hypertrophied nuclei) x 200.

F C.S. of hindgut in *Metapenaeus dobsoni* affected with WSD (ep-epithelial layer; teg-tegumental gland cell; arrows indicate basophilic inclusion bodies in the hypertrophied nuclei of the infected cells) x 200.

FIGURE 34



inclusions were seen in the hypertrophied nuclei of the hindgut epithelial cells. The size of the infected nuclei ranged from 5 μ to 14 μ in diameter. The lumen between the hindgut folds was also reduced as the large hypertrophied nuclei of the epithelial cells lining the lumen projected into it. The cuticular lining was also not clear. Here also, the infected nuclei were seen clustered together, as the affected cells lacked cell boundaries.

HEART

The heart was located ventral to the dorsal cephalothoracic cuticle and associated cuticular epidermis and dorso-posterior to the hepatopancreas. The heart was a triangular structure with its apex in front and base behind. It was suspended within the pericardium or pericardial chamber, at various points by suspensor ligaments. The pericardium was surrounded by the pericardial septum, which was normally thick and spongy dorsally and laterally, while thin and dense ventral to the heart. Immediately surrounding the heart was the protective epicardium, composed of a specialised spongy connective tissue. The heart was composed of myocardial cells, arranged in bands, which were primarily organised into distinct bundles. The bundled arrangement of the myocardium organised the heart lumen into subchambers. The nuclei of the myocardial cells are seen in fig. 35A, in which the nucleoli and nuclear membranes are quite distinct. Nuclei of the associated satellite cells were smaller than those of the myocardial cells and were seen scattered. The epicardium was composed of cells, which contained vesicular nuclei and wider area of cytoplasm. Five pairs of apertures or ostia pierced the muscular wall of the heart, each ostium being a small slit, the tips of which acted as valves. In the transverse section, the heart looked like a thick spongy meshwork of muscle fibres, the interstices of which represented the cavity of the heart. This cavity of the heart was not continuous, but was traversed by a

large number of interlacing muscle fibres, as observed in fig. 35A. The size of the normal nuclei ranged from 3.3 μ to 6.6 μ in diameter.

Structural disintegration was a noticeable feature of the heart in *Penaeus indicus*, affected with WSD. Most of the myocardial cells were affected. The hypertrophied nuclei contained eosinophilic to basophilic intranuclear inclusion bodies. The size of the hypertrophied nuclei ranged from 4 μ to 8.5 μ in diameter. Extensive melanisation and necrosis were noted in some muscle bundles. Large voids were also present in between the muscle fibres. A few satellite cell nuclei also showed signs of infection. Haemocytes observed in the affected areas exhibited basophilic, intranuclear inclusion bodies in them (Fig. 35B). Most of the affected nuclei were spherical in shape and lost their nucleoli. The entire hypertrophied nuclei were seen occupied by the inclusion bodies. Remnants of disintegrated tissues were also observed (Fig. 35C). In fig. 35D, margination of the chromatin as a ring-like structure near the nuclear membrane was well visible. A clear space could be seen surrounding the infected nuclei, especially around those containing basophilic inclusions. A few apparently normal nuclei, each with a distinct nucleolus and nuclear membrane were also observed.

In the affected heart muscles of *Penaeus monodon*, nuclei of the myocardial fibres and satellite cells were seen infected (Fig. 35E). Melanisation and severe necrosis of the muscle fibres were also noted. The size of the hypertrophied nuclei with eosinophilic to basophilic intranuclear inclusion bodies ranged from 4 μ to 7.6 μ in diameter. Very remarkable structural alteration and tissue disintegration were observed in the affected heart muscles of *Metapeaneus dobsoni* as shown in fig. 35F. Highly hypertrophied nuclei, with basophilic

Fig. 35. Light micrographs showing sections of heart in penaeid prawns

A C.S. of heart of normal *Penaeus indicus* (myo-myocardial cell; msb-muscle band; sat-nucleus of satellite cell; n-nucleus) x 200.

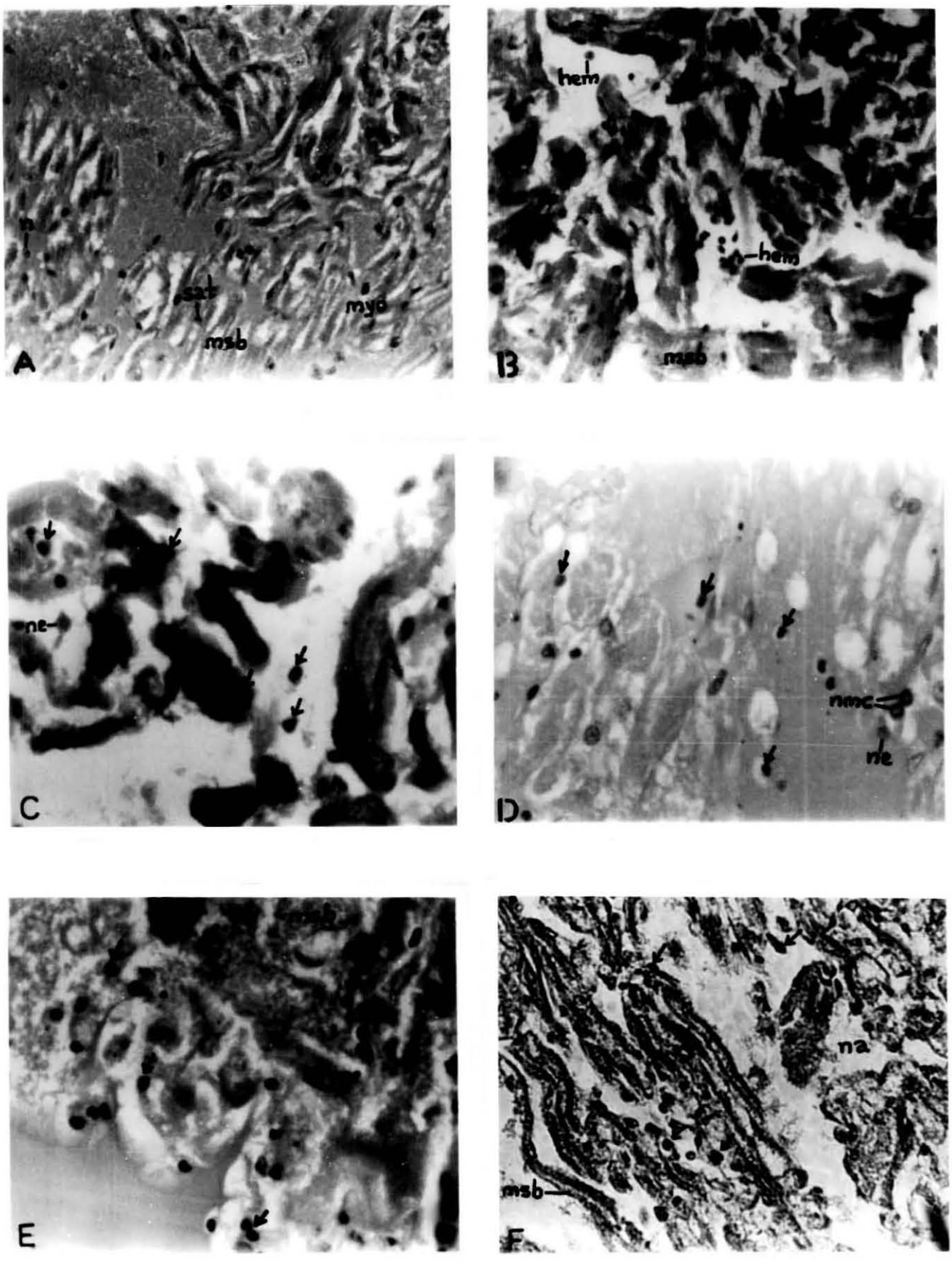
B C.S. of heart of *P. indicus* affected with WSD (msb-disintegrated muscle bundles; hem-haemocytes) x 200.

C & D Light micrographs showing enlarged views of the heart muscles of *P. indicus* affected with WSD (ne-eosinophilic inclusion bodies; mel-melanised areas of muscle bundles; nmc-margination of chromatin in affected nucleus; arrows indicate basophilic inclusion bodies in the hypertrophied nuclei) x 400.

E C.S. of heart tissue of *P. monodon* affected with WSD (msb-muscle bundles; arrows indicate basophilic inclusion bodies in the hypertrophied nuclei) x 400.

F C.S. of heart muscle of affected *Metapenaeus dobsoni* (msb-muscle bundle; na-necrotic area; arrows indicate hypertrophied nuclei of myocardial cells with basophilic intranuclear inclusion bodies) x 200.

FIGURE 35



intranuclear inclusions were also observed. Melanisation of the affected muscle bundles was heavy. The size of the hypertrophied nuclei ranged from 6.6 μ to 13 μ in diameter.

COMPOUND EYE

The compound eye of the penaeid prawns was attached to the head region, by a proximal stalk. The ommatidium had two regions, namely, the peripheral dioptric portion and the innermost ganglionic portion. The outermost layer of the dioptric portion of the ommatidium was the cuticle of the eye, followed by the epicorneagenous cells. Immediately proximal were the cone cells and their laterally displaced nuclei. Below, the cone cells were the quadripartite crystalline cones, characterised by the distal pigment granules, lying lateral to each crystalline cone as seen in fig. 36A. A major part of the ommatidia was composed of crystalline tracts, which formed the distal, lightly staining area, seen below the crystalline cone area (Figs. 36B & 36C). Next portion was the narrow layer of retinular cell nuclei, lying in close contact with the crystalline tracts. The diameter of the retinular cell nuclei ranged from 3.2 μ to 7 μ. Behind this was a darker, broader region of the striated rhabdomes. Retinular cell bodies were also seen in this region as seen in fig. 36C. The nuclei of the retinular cells lied distal to the cell bodies and in them, the centrally located nucleolus and nuclear membrane were seen distinctly (Figs. 36C & 36D). Pigment granules were also found, associated with the retinular cell bodies. A basement membrane divided this dioptric portion of the ommatidium from the inner ganglionic region. The retinular proximal pigment formed two bands below the basement membrane, the distal band and the proximal band, both of which lied within the fasciculated zone,

Fig. 36. Light micrographs showing sections of compound eye in penaeid prawns

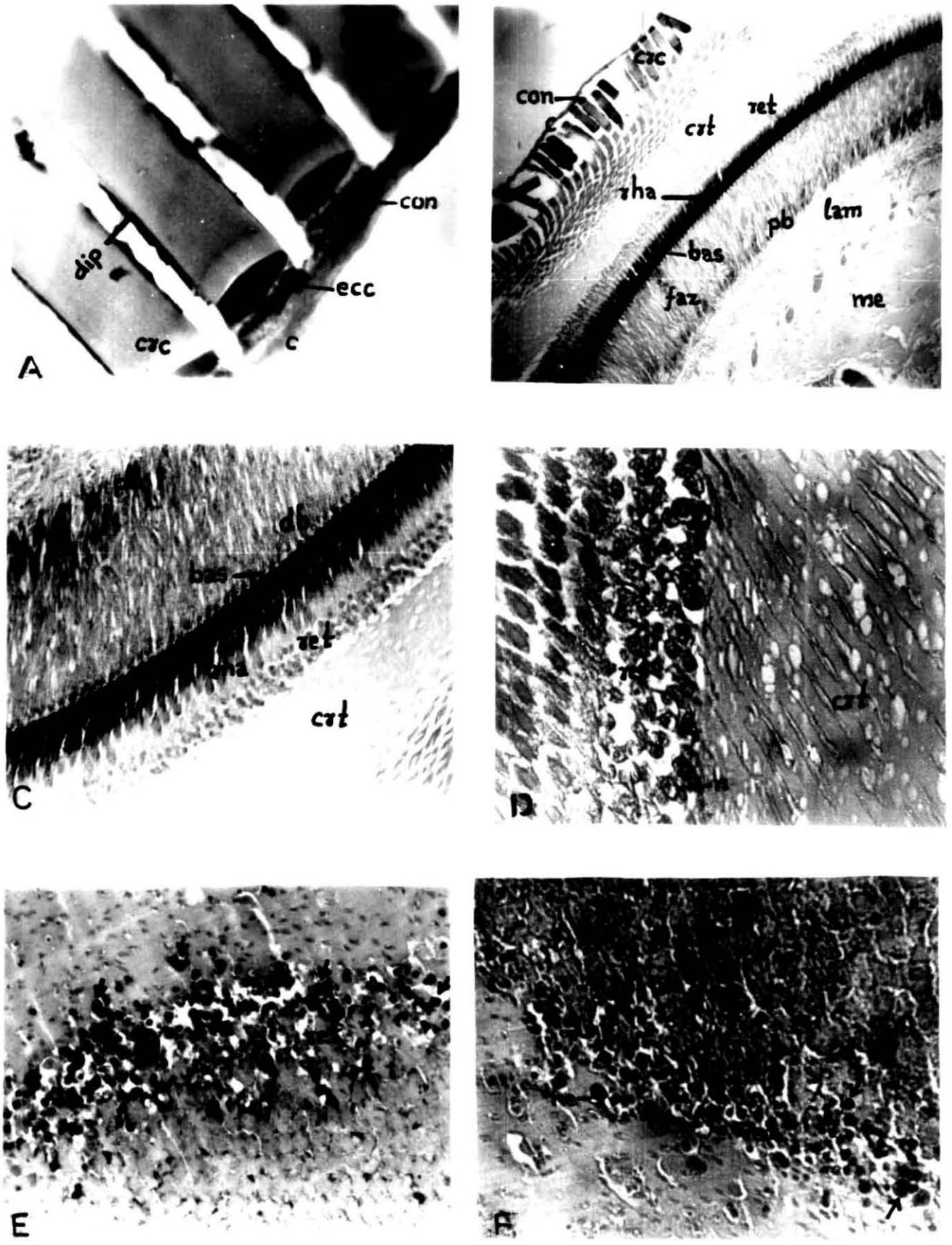
A Light micrograph showing the outer layers of compound eye in healthy *Penaeus indicus* (c- outerlaying cuticle; ecc-epicorneagenous cell; con-cone cell; crc-crystalline cone; dip-distal pigment granules) x 400.

B & C L.S. of the compound eye of normal *P. indicus* showing different layers in dioptric region (c-cuticle; con-cone cell; crc-crystalline cone; crt-crystalline tracts; ret-retinular cell nuclei region; rha-rhabdomes and retinular cell bodies; bas-basal membrane; faz-fasciculated zone; db-distal band; lam-lamina ganglionaris; me-medulla externa) x 40 (B), x 100 (C).

D An enlarged view of the retinular cell nuclei region (ret); crt-crystalline tracts; n-normal nucleus with a clear nucleolus. x 200.

E & F Light micrographs of the retinular cell nuclei region (ret) in *P. monodon* affected with WSD (ne-eosinophilic inclusion bodies; arrows indicate basophilic inclusion bodies in the hypertrophied nuclei of retinular cells) x 200.

FIGURE 36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which also contained primary optic nerve fibres. All these different layers could be clearly seen in fig. 36B.

In heavily infected *Penaeus monodon*, histopathology of ommatidium revealed the presence of isolated or grouped, very large and highly basophilic intranuclear inclusion bodies in the hypertrophied nuclei of the retinular cells, with large voids in between. Whereas, in the control, the nuclei in the retinular cells nuclei region were tightly packed with no empty spaces in between. The nuclear membranes and nucleoli disappeared in the infected nuclei of the retinular cells (Fig. 36E & 36F). The size of the nuclei varied between 3.3 μ and 14 μ. In the advanced stages of infection, the entire retinular cell nuclei area was seen occupied by basophilic inclusions and this portion became very distinct, as it stained deeply with H & E .

A few nuclei in the distal band of the retinular proximal pigment area of the fasciculated zone were also observed to exhibit the characteristic features of WSBV infection, such as hypertrophy and basophilic nature. The overlying cuticle was seen widely separated from the cone cells, as observed in fig. 37A. Displacement and disintegration of both the cone cells and crystalline cone were also observed. In such regions, the cytoplasm and the nuclei of the epicorneagenous cells were not clear. Immediately below the outermost cuticular layer, large voids were seen as in fig. 37A. Both the infected and apparently normal nuclei were observed together in the retinular cell nuclei region (Fig. 37B). In them, the inclusion bodies were small and mostly eosinophilic, compared to the large, highly basophilic inclusion bodies, seen in the heavily infected organisms (Fig. 36E). In some nuclei, a clear region was observed between the intranuclear inclusion body and the surrounding nuclear membrane.

Histopathological observations of the compound eye from WSD infected *Penaeus indicus*, revealed eosionphilic to basophilic, intranuclear inclusion bodies in the hypertrophied nuclei of the retinular cell bodies in the dioptric region of the ommatidia. Nucleoli and nuclear membranes were not seen. Though, hypertrophied nuclei, with the inclusion bodies differed in shape, generally they appeared spherical. Both eosinophilic and highly basophilic inclusions were visible in this affected region (Figs. 37C & 37D).

Compared to the infected *Penaeus monodon* and *P. indicus*, the number and concentration of the intranuclear, basophilic inclusions in the nuclei of the retinular cell bodies of the ommatidia of the WSD affected *Metapenaeus dobsoni* were less (Fig. 37E & 37F). In *M. dobsoni*, the striated nature of the rhabdome and retinular cell bodies were quite clear. Both eosinophilic and basophilic inclusions were seen in the nuclei of the retinular cells. The sizes of the infected nuclei ranged from 3 μ to 10 μ in diameter. Vacant spaces were present in between the crystalline tract and retinular cell nuclei area. A few apparently normal nuclei were also seen in between the affected nuclei, each with a distinct nucleolus and nuclear membrane.

In none of the above studied target tissues, occlusion bodies or cytoplasmic inclusions were observed.

Fig. 37. Light micrographs showing sections of compound eye of penaeid prawns affected with WSD

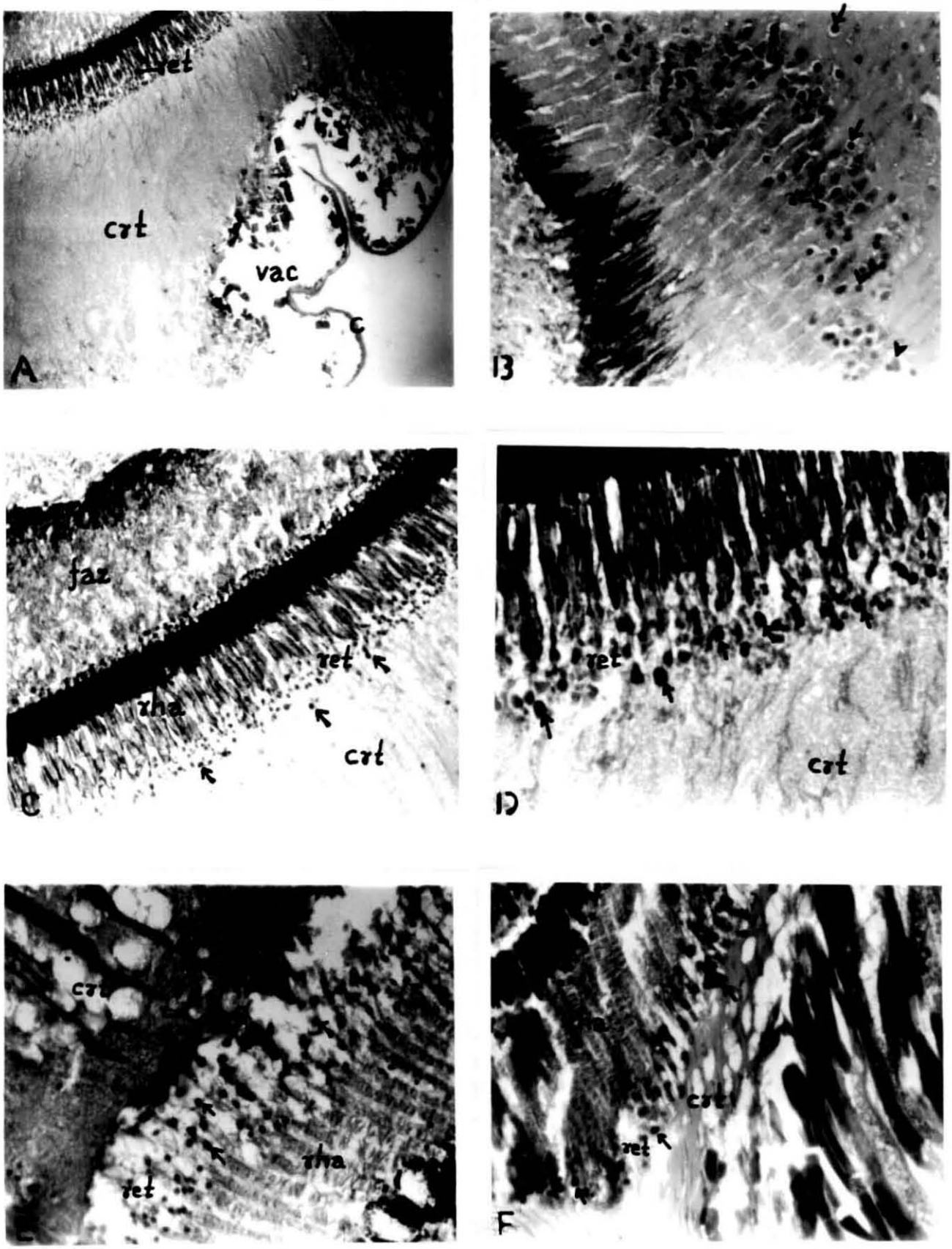
A Light micrograph showing disintegration and necrosis in the subcuticular layers of compound eye in affected *Penaeus monodon* (vac-vacant areas; crt- crystalline tract; c-cuticle) x 40.

B Light micrograph showing early stages of infection in the retinular cell nuclei region (ret) of *P. monodon* affected with WSD. Arrows and arrow heads indicate basophilic and eosinophilic inclusion bodies respectively in the hypertrophied nuclei. x 200.

C & D Light micrographs showing the affected retinular cell nuclei region (ret) in the compound eye of *P. indicus* infected with WSD (crt-crystalline tract; rha-rhabdomes and retinular cell bodies; faz-fasciculated zone; arrows indicate eosinophilic to basophilic inclusion bodies in the hypertrophied nuclei of retinular cells) x 100 (C), x 200 (D).

E & F Light micrographs showing basophilic inclusion bodies, indicated by arrows in the hypertrophied nuclei of the retinular cells in the ommatidium of *Metapenaeus dobsoni* affected with WSD (crt-crystalline tracts; rha-rhabdomes and retinular cell bodies) x 200.

FIGURE 37



2.4 DISCUSSION

Histopathology of different tissues, such as subcuticular epithelium, gill epithelium, foregut, hindgut, heart and compound eyes from *Penaeus indicus*, *P. monodon* and *Metapenaeus dobsoni*, affected with white spot disease (WSD) was studied. Along with this normal histology of all these tissues from healthy prawns had been done to make a comparative study and the results obtained are discussed here.

The different layers of the cuticle of *Penaeus indicus* consisted of epicuticle, exocuticle and the innermost membranous layers which are typical for all the normal penaeid prawns. The columnar epithelial cells had well defined, basally located nuclei with typical features of normal cell nuclei, such as nuclear membranes and nucleoli. Bell and Lightner (1988) had described the cuticular structure of normal *P. monodon*, which also consisted of an outer epicuticle, middle exocuticle and an inner layer. The present observations are in agreement with the observations of the above cited authors. The connective tissue layer, seen below the epidermis and the tegumental glands embedded in them were quite intact, having structural and functional integrity. The picture was very similar to the one described by Patwardhan (1958) in normal *Palaemon sp.* All these cells had well defined nuclei and the ducts of the glands led to the cuticular surface. The secretions of these glands were emptied to the exterior of the prawn's cuticle, as a result of which the natural glow and smoothness of the cuticular surface was maintained. Moreover, the mucus, secreted by these glands contained microbicidal agents, which offered resistance to the prawns, and the natural health was retained. Pigment containing bodies were observed in the exocuticle. Bell

and Lightner (1988) also described such chromatophores and stated that they were responsible for the colouration of the prawn.

In white spot disease (WSD) affected *Penaeus indicus*, the component layers of the cuticle were not distinct and they had a granular consistency. No such report existed and hence the present observation is of significance. The cuticle was very loose and easy to detach from the underlying epidermis in the diseased specimens. This characteristic of WSD affected specimens was reported by Durand *et al.* (1996) and Lo and Kou (1998). The artifactual separation seen between the cuticle and the underlying epidermis was more pronounced in the affected specimens than that in the normal penaeid prawns in the present study. This indicated the separated cuticle in the affected prawns.

The cuticular epithelium of WSD affected *Penaeus indicus* showed marked histopathological changes. Hypertrophied nuclei of the WSD affected cuticular epidermal cells, which contained eosinophilic to basophilic intranuclear inclusion bodies, were the most striking feature of the disease. In all these affected nuclei, the normal, functionally active components, such as the nuclear membrane, nucleoli etc, were not present. Eosinophilic inclusions, observed in the present study indicated the early stages of infection. The highly basophilic nature of the inclusion bodies in the advanced stages of infection was due to the accumulation of more viral particle. According to Chang *et al.* (1996) and Durand *et al.* (1996) the cuticular epidermis from every part of the body of the WSD affected shrimp was one of the main target tissues for WSBV. Momoyama *et al.* (1994, 1997), Chou *et al.* (1995) and Wang *et al.* (1995, 1997b) also supported this observation and opined that the cuticular epidermis in penaeid prawns was the most heavily affected of all the tissues examined. Supamattaya *et al.* (1998)

observed hypertrophied nuclei in subcuticular epithelial cells of the krill, *Acetes sp.* experimentally infected with WSSV. Tissues, showing such hypertrophied nuclei, characteristic of WSBV infection gave positive *in situ* hybridisation reaction with the probe. The differential staining of the early infected nuclei, as observed in the present study, with eosinophilic intranuclear inclusion, surrounded by marginated basophilic chromatin was reported by Kasornchandra *et al.* (1998) in the cuticular epithelium of *P. monodon* affected with WSD. Haemocytes and connective tissues were also seen affected by WSSV in the present study, indicating their mesodermal origin. Durand *et al.* (1996) and Supamattaya *et al.* (1998) detected WSBV positive haemocytes and connective tissue cells in P. *vannamei, P. stylirostris, P. monodon*, krill (*Acetes sp.*) and crabs. Enlarged vacant areas, noticed in the affected epidermal tissues were probably due to the disintegration and lysis of nuclei and cells. The sizes of the hypertrophied nuclei, observed in the present study were 2 to 4 times greater than those of normal nuclei. Lo *et al.* (1997) also reported similar size range for hypertrophied nuclei in the WSD affected cuticular epidermis. This increased size of the affected nuclei might be attributed to the enlargement of nuclei, in order to accommodate the large number of vigorously multiplying viral particles.

In *Penaeus monodon*, affected with WSD also, same pathological changes as observed in *P. indicus* were noted. The multifocal necrosis and cell lysis seen in the present case, were in agreement with the earlier studies made by Kasornchandra *et al.* (1998), who examined similar necrosis and lysis in the epidermal cells of *P. monodon* affected with WSD. The clustering of the hypertrophied, highly basophilic nuclei, observed were probably due to the disintegration of the adjacent cell membranes and organelles. Some hypertrophied nuclei occupied the entire cell, replacing the cytoplasm. This was observed by

Kasornchandra *et al.* (1998). Structural abnormalities, such as bulging of the particular area of the cuticular epidermis was observed in the present study, as the highly hypertrophied nuclei clustered together and exerted pressure on the overlying cuticle. Structural alterations were observed in the tegumental glands. Secretory functions of these glands were affected. This was quite evident from the roughness of the cuticular surface in the diseased prawns. The heavy infestations of epicommensals found in the present study could be probably traced to the fact that loss of secretions might have favoured attacking of epicommensals. Murali Manohar *et al.* (1996), Wang *et al.* (1997b) and Supamattaya *et al.* (1998) stated that *P. monodon* affected with WSD had epicommensal infestation and *Vibrio* infection.

WSD affected prawns exhibited small white spots on the inner surface of the cuticle. They are more clear and numerous in the advanced stages of infection. In the opinion of Wang *et al.* (1997b), these white spots were derived from the abnormalities of the cuticular epidermis. Chang *et al.* (1998) also reported that the production of white spots required the cuticular epidermis to become highly infected with virus.

In the present study, cuticular epithelium, covering the antennae and pleopods also exhibited hypertrophied nuclei with basophilic intranuclear inclusions. In many cases rupture of antennae were noticed. In severe cases, cuticular epidermis of antennae lost its structure. Hypertrophied nuclei with basophilic inclusion bodies were the only remnants of the cells. Lo *et al.* (1997) and Wang *et al.* (1999) reported basophilic inclusion bodies in the cuticular epidermis of pleopods, gills, stomach, pereiopods and eyestalks. But they did not report the occurrence of inclusion bodies in the subcuticular epithelium of

antennae, affected with WSD, since in severe cases antennae was lost and probably they could not observe the inclusion bodies in them. In almost all the affected specimens, broken antennae were a characteristic feature and this was probably due to the damaged, subcuticular epithelium of the antennae. Moreover, the antennal glands were also reported to be affected with WSBV in marine prawns (Momoyama *et al.*, 1994; Chang *et al.*, 1996, 1998; Wang *et al.*, 1997b, 1999). In cases of severe infection, the cuticular epidermis of the antenna was completely disintegrated and hypertrophied nuclei with basophilic intranuclear inclusions were observed in the space between the cuticle and the underlying epidermis. This could be due to the rupture and disintegration of the epidermal cells.

Cuticular epidermis of the antennae of *Metapenaeus dobsoni*, collected from WSD affected prawn farms also, exhibited symptoms of the disease. Such an observation is very rarely reported. One of the first and important symptoms of WSD, observed by many farmers was the failure in moulting. This could be attributed to the over all structural and functional disorders in the different layers of cuticle and sub cuticular epithelium of penaeid prawns, affected with WSD.

As the gills were constantly in direct contact with the aquatic medium, the cuticular lining present in the periphery protected the thin epithelial cells, lying beneath. Structure of the gill in *Penaeus indicus* observed in the present study revealed the presence of afferent and efferent vessels in the basal region of the primary filaments which helped in the gaseous exchange. Haemal sinuses were also present having circulating haemocytes. Pillar cells were very large and seen in between the adjacent epithelial cell layers in the gill filaments. Both fixed and circulating haemocytes were also observed. All these cells possessed the typical

structure, each with a nuclear membrane and a clear nucleolus. The cuticular lining and the haemocytes in the healthy prawns, played an important role in their defence mechanisms and protected them from opportunistic pathogens and other environmental pollutants. The structure and organisation of the gill filaments noticed in the present study were in agreement with the observations made by Bell and Lightner (1988) in normal *P. monodon*.

In the present study, subcuticular epithelial cells, haemocytes and pilaster cells had the inclusion bodies indicating viral infection. However, severe cellular degeneration and necrosis were observed mostly in the subcuticular epithelium. Kasornchandra *et al.* (1993), Wongteerasupaya *et al.* (1995a) and Lightner *et al.* (1998) reported that affected epithelial cells underwent degeneration and development of eosinophilic to basophilic inclusion bodies in them. The changes observed in the present study were also in confirmation with the observations of above cited authors. The degree of nuclear hypertrophy and the changes in the staining reactions of the infected nuclei indicated the different stages of viral development and maturation, as reported by Kasornchandra *et al.* (1998). Chang *et al.* (1998) opined that gills are a good choice for WSBV diagnosis in decapods.

Structural disintegration of the entire gill tissue was observed in *Penaeus indicus*. Both the primary and secondary gill filaments were distorted greatly and the integrity was lost. Wongteerasupaya *et al.* (1995a) also stated that in the advanced stages of infection, the state of tissue disintegration was so extensive that no distinctive character could be attributed specifically to SEMBV. Wang *et al.* (1997b) reported cellular lesions and tissue disorganisation in the affected gills of *P.monodon* and *P. japonicus*, the damage becoming evident as the

infection advanced. Extensive abnormalities including cells with hypertrophied nuclei, chromatin margination and nucleolar dissociation as noticed by the above authors were observed in the present case also. Marked hypertrophy and highly basophilic intranuclear inclusions were observed in the sheath cells lining the afferent and efferent vessels of gill filaments. Wang *et al.* (1999) reported such histopathological changes in the sheath cells of subgastric artery in experimentally infected *Farfantepenaeus duorarum*. Such lesions were observed in the present study also.

The hyaline, clear area observed in the present study between the eosinophilic and slightly basophilic inclusion bodies and the nuclear membrane was reported by Wongteerasupaya *et al.* (1995a) also in the gills of *Penaeus monodon* affected with SEMBV. This transparent zone is an indication of the earlier stages of infection. In the later stages of infection, the inclusion bodies became larger, highly basophilic and the transparent zone between the intranuclear inclusions and the marginated chromatin disappeared, as the inclusions fully occupied the nucleus. Large, vacant spaces were observed in the affected tissues in the present case. Such changes were reported by Wongteerasupaya *et al.* (1995a) in the infected gill tissues of *P. monodon*. These vacant areas were probably due to the disintegration of the affected nuclei and cells, indicating the advanced and final stages of infection. These vacant areas in some gill filaments were so extensive that the components were no longer discernible, as in the present case.

As disintegration was observed in the cuticular lining of both the primary and secondary gill filaments of infected *Penaeus indicus*, there were chances for osmotic imbalances. This could also be due to structural and

functional disintegration of the subcuticular epidermis of the affected gill filaments. The role of the thin, membranous layer of the cuticle and the underlying epidermal tissue in the osmoregulation of crustaceans was well explained by Robertson (1960). The cuticular disruption, observed in the gills of infected *Penaeus indicus* in the present study was not reported earlier in WSBV affected prawns.

In the present study, certain giant cells with pale basophilic cytoplasm were observed in the haemal sinuses of the gills of one of the affected specimens of *Penaeus indicus*. There were both spherical and spindle shaped cells, with marginated, highly basophilic nuclei in them. Lightner (1996) also observed a unique spherical cell type with palely basophilic cytoplasm and central spherical nucleus in the haemal sinuses of gills, hepatopancreatic tubules, heart and antennal gland in prawns with yellow head disease. These cell types were thought to be immature haemocytes, released prematurely from the haematopoietic organs in response to YHV induced haemocytopenia. In the present case, the nuclei in the infected cells were laterally displaced. Similar giant cells were reported by Mohan and Shankar (1999) from the cultured *Penaeus monodon*, showing gross signs of white spot syndrome (WSS) and yellow head syndrome (YHS), during the 1994 Indian epizootic. They observed unusually large cell types with a central vacuole and a marginated basophilic nucleus in the heart tissues of the affected prawns. According to Mohan and Shankar (1999), perhaps, these giant cells would eventually be found to be characteristic of dual YHV/WSSV infections. The origin and function of these giant cells were not clear. From their unusual size, round shape and distortion of the nuclei, they seemed to have a phagocytic function. Wang *et al.* (1999) observed fusion of haemocytes to form multinucleated giant cells in the antennal glands of

Litopenaeus vannamei and *Farfantepenaeus duorarum*, infected with WSBV. Such fusion of cells was not recorded in the present work.

Histopathological changes and eosinophilic to basophilic inclusion bodies, similar to those observed in *Penaeus indicus*, affected with WSD were noted in the affected *Penaeus monodon* also. All the observations made in the present study on *P. monodon*, affected with WSD, perfectly agreed with the reports made by Wongteerasupaya *et al.* (1995a), Lo *et al.* (1997), Wang *et al.* (1997b) and Kasornchandra *et al.* (1998) in the gill tissues of infected *P. monodon*. According to Chang *et al.* (1996), the early target organs of WSBV in *P. monodon* included the gill and cuticular epidermis. The sizes of the hypertrophied nuclei in the affected gills of *P. indicus* and *P. monodon*, observed in the present study, were similar, ranging from 3.3μ to 13μ in diameter. Sizes of the abnormal nuclei differed, depending on the origin of the cells and the degree of infection. Momoyama *et al.* (1997) reported a size range of 7 μm to 13 μm for the hypertrophied nuclei of various tissues of ectodermal and mesodermal origin in *Metapenaeus ensis* affected with PAV.

In the gill filaments of *Penaeus monodon*, affected with WSD, the different stages of inclusion bodies could be observed. Both eosinophilic and basophilic inclusion bodies were seen in the same filament. The transition from eosinophilic to basophilic staining character could be well discerned. Similar observations were made in *P. monodon* gill tissues, affected with SEMBV by Wongteerasupaya *et al.* (1995a) and Murali Manohar *et al.* (1996).

In the present study, the foregut, hepatopancreas, midgut and hindgut of virus free *Penaeus indicus* agreed with that of *P. monodon*, described and

explained by Bell and Lightner (1988). In the present investigation, the epithelial layer, lying below the cuticle showed large number of intranuclear inclusion bodies in them. Basophilic intranuclear inclusion bodies were observed in the hypertrophied nuclei of the spongy connective tissue also. Chang *et al.* (1996), Durand *et al.* (1996), Lo *et al.* (1997), Kasornchandra *et al.* (1998), Lightner *et al.* (1998) and Wang *et al.* (1999) observed similar histopathological changes in the subcuticular epithelial cells of the stomach of penaeid prawns. Chang *et al.* (1998) opined that stomach epidermis was one of the most severely infected organs in the marine shrimps, affected with WSD. Rajendran *et al.* (1999) observed more prominent, deeply basophilic, large and greater number of WSSV inclusions in the gut wall of experimentally infected mud crabs, prawns and lobsters. Chang *et al.* (1998) detected WSBV DNA in the stomach of freshwater shrimps (*Macrobrachium sp.* and *Procambarus clarkii*).

In the present study, infected nuclei of the epithelial cells of the stomach of *Penaeus indicus* were highly hypertrophied, ranging in size from 6.6 μm to 13.5 μm in diameter. Chang *et al.* (1996) also opined that in the affected epithelial cells of stomach, some cell nuclei were enlarged to more than twice the diameter of the normal nuclei. Empty spaces, observed in between the epithelial cells in the present study indicated severe necrosis and cell lysis due to viral infection. Durand *et al.* (1996) also observed similar necrotic areas in the stomach epithelium of affected *P. monodon* and reported accumulation of infected haemocytes in the connective tissues. Such, clustering of infected haemocytes was noticed in the present study also. There was a possibility of both the fixed and circulating haemocytes to be infected by WSBV. However, the haemocytic aggregations found in association with epithelial necrosis of the affected stomach were confirmed to be infected circulating haemocytes by Durand *et al.* (1996).

Affected nuclei were present in a line in the stomach epithelium, without any cell boundaries or other organelles. There was a possibility of releasing these inclusion bodies into the lumen of the stomach by the rupture of the epithelial cells, which could re infect fresh epithelial cells. This might come out through the faecal matter and could cause fresh infection. It was found that occlusion bodies of MBV were released through the faeces of prawns and this led to further infection of prawns (Lightner, 1993; Karunasagar *et al.*, 1998). But this is not yet confirmed in the case of WSBV and this aspect needs further study.

The empty stomach, noticed in almost all the affected *Penaeus indicus* specimens, without any remnants of food material could be a clear evidence of starvation and functional disintegration of various cell types. A few basophilic inclusions were seen scattered in between the inter setal grooves also, which could have been released from the severely affected epithelial cells by the disintegration of the cell boundaries. Based on an experiment conducted, Chang *et al.* (1996) opined that infection might occur through the oral route. According to them, there were chances of infection, if the virus containing food just entered the stomach lumen and some of it adhered to the setal grooves of the cuticular layer, or might also resulted from a non specific binding to chitin, as had been reported by Lightner (1996). This was based on the observation that a few positive signals could be detected in the cuticular layer above the stomach epithelium by *in situ* hybridisation, using probes. Highly basophilic inclusion bodies were observed in the sub cuticular epithelium of hindgut in penaeid prawns affected with white spot disease in the present investigation. Similar report was made by Jasmin and Mary (2000) in the hindgut epithelium of affected *Penaeus indicus.*

The granular material found in the lumen of the hepatopancreatic tubule was the secretory product of the tubular epithelial cells, especially B-cells. This indicated the functionally active stage of the organ. The spongy connective tissue sheath, presented in the peripheral area of the hepatopancreatic lobes had normal nuclei. Other tubular epithelial cells, such as the E-cells, F-cells etc. were also well distinguished. The structure of the normal hepatopancreas of *Penaeus indicus* observed in the present study was similar to the structure described by Bell and Lightner (1988) in normal *P. monodon.*

In the hepatopancreas of WSD affected *Penaeus indicus*, the tubular epithelial cells were not affected and were seen to be intact. They exhibited normal nuclei and other cellular characteristics. But the haemocytes present in the haemal sinuses and the connective tissue layer showed hypertrophied nuclei with intranuclear inclusion bodies in them. However, the intensity of infection was low and the inclusion bodies observed were mostly eosinophilic or pale basophilic. Nuclear hypertrophy was also not distinct. But the infected nuclei stained homogeneously with H & E. Lo *et al.* (1997) reported that in hepatopancreas and other lightly infected target organs in *P. monodon*, the infected nuclei were not obviously hypertrophied and remained almost close to the normal size and so they were not easily distinguishable. This observation agreed with our present observation. The organ integrity was maintained. According to Chang *et al.* (1996, 1998), Durand *et al.* (1996) and Lo *et al.* (1997), in the hepatopancreas of *P. monodon*, affected with WSD, a few WSBV positive cells were located in the fibroblasts of connective tissue sheath and the myoepithelial cells of the hepatopancreatic sheath. Lo *et al.* (1997) strongly argued that hepatopanceatocytes were never found to be WSBV positive. Supamattaya *et al.* (1998) also did not get any marked pathological changes in the hepatopancreas of

krill, affected with WSD. Wang *et al.* (1997b) reported WSD virus infected haemocytes in the hepatopancreatic intertubular spaces in *P. monodon* and *P. japonicus*, affected with WSD. The present observation also indicated that heaptopancreatic cells were refractive to WSSV infection. However, Durand *et al.* (1996) could get positive labelling of the apical part of columnar epithelial cells of the hepatopancreatic tubules by immunofluorescent assay. They stated that this might be due to absorption of viral particles released from infected cells or by ingestion of infected animals. Present result agreed with the observation of Durand *et al.* (1996)

In the midgut epithelium also, no signs of WSBV infection were observed. Durand *et al.* (1996), Lo *et al.* (1997) and Lightner *et al.* (1998) observed the same in the epithelial cells of midgut in WSD affected penaeid prawns. However, Chang *et al.* (1996) and Lo *et al.* (1997) reported some WSBV positive connective tissue cells and muscle cells in the midgut. Heavy infection of haemocytes in the haemal sinuses was noticed in the present case and this observation was in agreement with the reports of Durand *et al.* (1996). According to the authors, many fixed and circulating haemocytes showed a positive reaction with WSBV probe and these were observed throughout the haemocoel and in the haemal sinuses of the hepatopancreas and amongst the striated skeletal muscles. In the present case, the gastric arteries in the affected *P. indicus* revealed severe infection and were densely packed with highly basophilic haemocytes, having hypertrophied nuclei, which almost filled the entire cell. Similar observation was made by Wang *et al.* (1999) and according to them, the subgastric artery appeared to be one of the target tissues, most severely affected by WSSV in *Farfantepenaeus duorarum*. Hepatopancreas and mid gut were refractive to WSSV infection. These two organs are of endodermal origin. It was seen that

white spot syndrome virus grew only in ectodermal and mesodermal tissues (Wongteerasupaya *et al.*, 1995a; Durand *et al.*, 1996; Lo *et al.*, 1997; Wang *et al.*, 1997b; Lo and Kou, 1998). This would be the reason for the non-occurrence of WSSV in the hepatopancreas and midgut cells in the present study.

The epicardium, myocardial cells and the chambered nature of the heart were well visible in the control *Penaeus indicus* in the present study. In the normal, healthy prawns, circulating and fixed haemocytes were also seen. The structure of the heart of *Penaeus indicus*, described in the present study agreed with the description given for normal *P. monodon* by Bell and Lightner (1988). The lips of the ostia, which acted as valves helped in allowing hemolymph to flow only in one direction, i.e., from the surrounding pericardial sinus into the heart and not vice versa.

Heart, one of the most important vital organs, in penaeid prawns is easily prone to WSSV infection. Of the various cell types present in the heart of *Penaeus indicus*, myocardial cells and haemocytes showed inclusion bodies indistinguishable from WSSV inclusions. These cells exhibited eosinophilic to basophilic inclusions in their hypertrophied nuclei. Few number of satellite cells were also affected with virus. Similar observations were made by Murali Manohar *et al.* (1996), Momoyama *et al.* (1997) and Chang *et al.* (1998). According to Lo *et al.* (1997), some myocardial cells were WSBV positive with *in situ* hybridisation, but the epicardial cells were WSBV negative. Lo *et al.* (1997) and Peng *et al.* (1998a) also reported WSBV positive haemocytes in the heart lumen. Chang *et al.* (1996) detected positive cells in the muscle fibres and connective tissues of the heart in *P. monodon* affected with WSD.

In the present study, normal nuclei in the heart tissues showed a size range of 3.3 μm to 6.6 μm in diameter, whereas, the infected nuclei exhibited 4 μm to 8.5 μm in diameter in *Penaeus indicus* and 4 μm to 7.6 μm in diameter in *P. monodon* affected with WSD. From this, it was very clear that there was not much difference in the size of the nuclei in the control and in the affected specimens. In the heart tissue, hypertrophy of the affected nuclei was not so evident, as that in other tissues like subcuticular epidermis, gills and gut. But the nuclei stained homogeneously with H & E. Lo *et al.* (1997) also reported that the nuclei of the abdominal muscle, heart, lymphoid organ, midgut, nervous tissues, hepatopancreas, testes, ovaries and spermatophores in infected *P.monodon* were not so obviously hypertrophied and remained close to the normal size and so, they were not readily distinguishable. In their studies, these affected nuclei stained uniformly with H & E staining. Whereas in other tissues, such as cuticular epidermis, gills etc., the diameters of the hypertrophied nuclei were two to three times larger than those of the normal nuclei. Margination of the chromatin near the nuclear membrane and eosinophilic inclusions were indications of early infection (Wongteerasupaya *et al.*, 1995a). In the advanced stages of infection, the entire hypertrophied nuclei were seen occupied by the highly basophilic intranuclear inclusion bodies and the nuclear membrane and nucleoli were no longer present. Large voids, observed in the present case were due to the disintegration and lysis of the affected nuclei and cells. The circulating haemocytes were more in the haemolymph. As heart acted as a receiver for large volumes of haemolymph, the chances for mutual infection were more.

Observations made in the infected heart of *Penaeus indicus*, such as slight nuclear hypertrophy, intranuclear inclusion bodies of varying staining reactions and melanisation of the affected necrotic areas, mainly myocardial cells

and satellite cell areas were seen in *P. monodon* heart tissue also. Murali Manohar *et al.* (1996) made similar observations in the heart muscles of *P. monodon* affected with SEMBV. In the heart of *Metapenaeus dobsoni* also, structural disintegration and presence of intranuclear inclusion bodies were noted. Hypertrophy of the affected nuclei and melanisation of the necrotic areas were a common feature. But the histopathological changes were not so extensive and severe as in the case of *P. indicus* and *P. monodon*, affected with WSD. Such observations, noted in the heart of affected *M. dobsoni* have not been reported so far.

In the control prawns, the various component layers of ommatidium, such as, the outermost cuticle, epicorneagenous cells, cone cells, crystalline tracts, retinular cells, fasciculated zone and the ganglionic regions were intact and clearly visible in the L.S. of the ommatidia. The retinular cell nuclei region, the area of great interest in the present study, was a lightly stained, faint portion with very distinct nucleoli and nuclear membrane. The structure of compound eyes of normal penaeid prawn observed in the present case was exactly similar to that described by Bell and Lightner (1988) in *Penaeus monodon.*

Histopathology of the affected ommatidia of *Penaeus monodon* revealed nuclear hypertrophy and eosinophilic to basophilic intranuclear inclusion bodies in the retinular cells nuclei in the dioptric portion. The entire retinular cell nuclei region appeared as a darkly stained, highly distinct area in the L.S. of the ommatidia. The eosinophilic inclusion bodies indicated the earlier stages of infection. Whereas, the highly basophilic, inclusion bodies occupied the entire, highly hypertrophied nuclei. Nucleoli and nuclear membranes were no longer present, as in the affected nuclei of other infected tissues, such as cuticular and gill epithelium, foregut, hindgut and heart. The transition stage between apparently

normal nuclei and infected ones, having both eosinophilic and lightly basophilic inclusion bodies were observed in the initial stages of infection. Wongteerasupaya *et al.* (1995a) also made similar observations in the gill epithelium of *P. monodon* and opined that in later stages of development, these inclusions were larger and their staining reaction changed from distinctly acidophilic to lightly basophilic with H & E. Jasmin and Mary (2001) made similar reports in the retinular cell nuclei region of *P. indicus* affected with WSD. The entire region was so extensively affected that no single apparently normal nucleus could be seen in between.

The cell layers below and above the retinular cell nuclei region appeared to be normal, except for some vacant spaces observed in the crystalline tract area. Chang *et al.* (1996) reported the fasciculated zone and the lamina ganglionaris of the compound eyes and the glial cells of the ganglia to be viral DNA positive at 40h pi in *P. monodon* by *in situ* hybridisation. According to them, these two regions were lightly infected by WSBV and the most severely affected site of the eyes was the cuticular epidermis of the eyestalk. Changes found in WSBV infected shrimps could be distinguished easily from the white eye syndrome in Taiwan in 1991, in terms of brown fibrous nodules seen in the fasciculated zone of compound eyes (Chen *et al.*, 1991; Chang *et al.*, 1996). Lo *et al.* (1997) also observed lightly acidophilic to highly basophilic, hypertrophied nuclei in the cuticular epidermis of the eyestalks with diameters, two to three times greater than those of normal nuclei. However, Lo *et al.* (1997) and Lo and Kou (1998) reported that eyestalks with compound eyes still attached produced consistently negative results with PCR. Lo *et al.* (1997) opined that this failure of amplification was due to the compound eye, which might have contained inhibitors of polymerases. Chang *et al.* (1998) also noted WSBV DNA positive

cells in the eyestalks of experimentally infected marine prawns. Sahul Hameed *et al.* (1998) reported that filtrates of eyestalk caused 100% mortality in an experimental infection trail in *P. monodon* and *P. indicus*.

The transparent zone or halo observed in between the intranuclear inclusion bodies and marginated chromatin in the present case. Such change was reported by Wongteerasupaya *et al.* (1995a) in the nuclei of the epithelial cells of the gill tissue in affected *P. monodon*. According to them, this was an indication of the early stages of viral infection, whereas, in the advanced stages, this transparent zone was no longer present. The degree of nuclear hypertrophy also varied with the stages of infection and viral development. This was clearly pin pointed in the present study, where the size of the retinular cell nuclei in lightly affected specimens ranged from 3.3 μm to 9 μm and that of the heavily infected specimens were 5 μm to 14 μm in diameter. A few nuclei in the proximal part of the fasciculated zone also exhibited intranuclear inclusion bodies, as observed by Chang *et al.* (1996). Structural alterations were observed in the outermost cuticular lining and epicorneagenous cell layers also. Similar histopathological changes were observed in the retinular cell nuclei of the ommatida of affected *P. indicus* and *Metapenaeus dobsoni* also. Vacant spaces were present in this area in between the affected nuclei, indicating disintegration and lysis of severely infected cells. These types of lesions were observed by Wongteerasupaya *et al.* (1995a) in the epithelial cells of *P. monodon*. Chang *et al.* (1996) also reported extensive necrosis and lysis of the infected cells in various target organs in the advanced stages of infection. Jasmin and Mary (2001) reported similar observations in the compound eyes of *P. indicus* affected with WSD.

The importance of the neurosensory photoreceptor cells in the retinular region of the compound eyes of the crustaceans and the major role played by them in the vision of the organism were described in detail by Shaw and Stowe (1982). The affected prawns in the present case exhibited disoriented swimming behaviour. It was noticed that the eyes of the affected prawns were pale, having being lost their natural glow. It was quite likely that, this might be due to the WSBV infection and the resultant structural and functional derangement of the retinular cell region of the compound eyes. So, the role played by the retinular cells in the behavioural changes of the affected prawns needs further investigation.

Histopathological changes observed in all the examined tissues, such as the subcuticular epithelium, gill epithelium, foregut and hindgut subcuticular epithelium, heart and compound eyes of three different penaeid prawn species, i.e. *Penaeus indicus, P. monodon* and *Metapenaeus dobsoni* were similar, though, the degree of infection varied with species. Compared to *P. indicus* and *P. monodon*, the rate of infection was low in *M. dobsoni*. The intensity of infection was noticed in the tissues of *P. indicus* and *P. monodon*, eventhough, the latter showed marked and severe external symptoms. This observation was supported by the results of the survey conducted by Lo *et al.* (1996) on the distribution of WSSV in wild penaeid shrimps of the adult stages, collected from the coastal waters around southern Taiwan. The study showed that detection rate of the virus in *P. semisulcatus* was much lower than that of *P. monodon* and *P. japonicus*. Sahul Hameed *et al.* (1998) compared the susceptibility of *P. monodon* and *P. indicus* to SEMBV infection and found that the former was more susceptible than the latter. However, SEMBV caused 100% mortality in both the species of prawns. Lightner *et al.* (1979), Lu *et al.* (1994) and Sahul Hameed (1995) had reported similar differences in susceptibility to viral, bacterial and fungal infection among penaeid

prawns. Based on an experimental infection trial carried out by Wang *et al.* (1998) with white spot baculovirus (WSBV) in some cultured and wild decapods in Taiwan, it was concluded that even within each infection type, different species exhibited various degrees of WSBV infection.

Eventhough, WSSV infections were limited to tissues of ectodermal and mesodermal embryonic origin (Wongteerasupaya *et al.*, 1995a; Durand *et al.*, 1996; Wang *et al.*, 1997b; Lo and Kou, 1998), the degree of infection varied greatly among the target tissues also. In the present investigation, the most severely affected tissues were the subcuticular epidermis, gill epithelium, stomach epithelium and the retinular cell nuclei region of the compound eyes. Heart tissue was the least infected tissue. In the opinion of previous workers, in WSD also, the severity of infection in different target organs differed greatly. According to Chang *et al.* (1996) and Wang *et al.* (1997b), cuticular epidermis, connective tissue and lymphoid organ were the major damaged target organs in WSD affected *P. monodon* and *P. japonicus*. Momoyama *et al.* (1994), Chou *et al.* (1995) and Wang *et al.* (1995) reported that WSBV most frequently attacked the cuticular epidermis. The major target organs of WSBV in penaeid prawns were connective and epithelial tissues, particularly epithelial cells of the gills and stomach (Inouye *et al.*, 1994; Anon., 1995; Wongteerasupaya *et al.*, 1995a). In the opinion of Chang *et al.* (1998), severe WSBV infection in crabs and lobsters occurred only in the gills and according to them gills were a good choice for WSBV diagnosis in decapods.

Histopathological studies of various tissues of *Metapenaeus dobsoni*, such as cuticular epidermis, hindgut, retinular cell nuclei of compound eyes and heart in the present study revealed the presence of WSBV infection. This was the

first report of *M. dobsoni* being affected with WSD, from the culture ponds, though, Rajendran *et al.* (1999) mentioned that they could induce WSD experimentally in *M. dobsoni*. Wang *et al.* (1997b) could detect WSD virus infection in the various tissues originated from ectoderm and mesoderm of wild caught greasy back shrimp, *Metapenaeus ensis* in Taiwan, a species belong to the same genera of the present study.

As with other baculoviruses, WSSV produced hypertrophied nuclei that had eosinophilic to basophilic inclusion with H & E staining and marginated chromatin. No cytoplasmic inclusions were found in the target tissues of WSSV. Wang *et al.* (1997a, 1997b) also did not find any cytoplasmic inclusion bodies in penaeid prawns affected with WSSV. Replication of WSBV appeared to be exclusively in the nucleus and occlusion bodies were not produced. Therefore, WSBV is closely related to the Baculoviridae family and to the non-occluded Nudibaculovirinae subfamily (Francki *et al.*, 1991). WSBV could infect shrimp tissues that originate from the ectoderm or mesoderm, similar to IHHNV and SEMBV. This distinguished WSBV from other shrimp baculoviruses, such as MBV, BP and BMNNV, which only infected the epithelial cells of the hepatopancreatic tubules and the midgut in prawns (Momoyama *et al.*, 1983; Chang *et al.*, 1992, 1993; Bruce *et al.*, 1993, 1994). Unlike other baculoviruses, WSBV and SEMBV caused systemic infection as they were distributed in organs of ectodermal and mesodermal origin. The target organs of WSD virus were similar to those of IHHNV (Lightner, 1983), yellow head virus (Chantanachookin *et al.*, 1993), RV-PJ (Inouye *et al.*, 1994) and SEMBV (Wongteerasupaya *et al.*, 1995a). The WSVB and SEMBV also differed significantly from other systemic baculoviruses and rod shaped virus reported by Boonyaratpalin *et al.* (1993), Chantanachookin *et al.* (1993) and Owens (1993) in morphology and size.

Although, YHD virus was rod shaped, it might be distinguished from WSD virus by the presence of cytoplasmic, basophilic inclusions in the YHD infected cells (Wongteerasupaya *et al.*, 1995b; Wang *et al.*, 1997b).

SEMBV inclusions, differed from that of other baculoviruses, in the early development, since they were surrounded by a clear zone (in Davidson's fixed tissues) and appeared similar to the Cowdry A-type inclusions, seen with infections of IHHNV (Lightner, 1988; Wongteerasupaya *et al.*, 1995a; Kasornchandra *et al.*, 1998). But in white spot syndrome, the Cowdry A inclusions represented an early stage of viral infection. Unlike, IHHNV, the WSSV infected nuclei undergo further degeneration and the inclusions change from eosinophilic to basophilic with age and the nucleus was surrounded by vacant cytoplasm. According to Wongteerasupaya *et al.* (1995a), these features could be used to distinguish infections caused by SEMBV or WSSV from those caused by IHHNV, which produced unchanging eosinophilic, nuclear inclusions, without vacant cytoplasm. Moreover, IHHNV belongs to the family, Parvoviridae (Bonami *et al.*, 1990). The histological profile of intranuclear eosinophilic inclusions, without the occluding protein, polyhedrin, was similar to that reported for non occluded baculovirus infections in penaeid prawns (Lihghtner, 1993). Another noticeable feature was that SEMBV infected prawns, did not show white spots in the carapace, which was the most prominent symptom of WSD affected prawns and other decapods reported so far. Based on the histopathological observations and external symptoms, such as the presence of small, obscure white spots on the carapace, WSDV was similar to RV-PJ virus in *P. japonicus*, as reported by Momoyama *et al.* (1994).

Eosinophilic to basophilic inclusion bodies were observed in tissues of ectodermal and mesodermal origin, such as, subcuticular epithelium of the exoskeleton, gills, stomach and hindgut and heart. In the compound eyes of affected penaeid prawns, retinular cell nuclei region exhibited the most pronounced symptoms of the disease. Loss of nucleoli and hypertrophy of the affected nuclei were other most significant characteristics of WSD infection. Cellular lesions, necrosis and disintegration of the tissues were also observed. The sizes of the hypertrophied nuclei ranged from 3 to 14 μm in diameter. Though, infected haemocytes and connective tissues were found in the hepatopancreas and midgut, these organs were observed to be non infected, as they were of mesodermal origin.

CHAPTER 3

ULTRASTRUCTURAL STUDIES

CHAPTER 3

ULTRASTRUCTURAL STUDIES

3.1 INTRODUCTION

Routine histology and transmission electron microscopy (TEM) were added to the diagnostic tools available to the early penaeid prawn pathologists by the mid 1970s. Couch (1974a, 1974b), for the first time used TEM to confirm the presence of *Baculovirus penaei* (BP) in the tetrahedral occlusion bodies observed during light microscopic examination of wet mounts of the hepatopancreas of Penaeus *duorarum* and *P. aztecus*. Thin sectioning method and electron microscopy allowed direct observation of virus-cell interaction, which in turn revealed the site of viral replication and maturation in the host cell, thus aiding identification of unknown viruses.

For rapid and confirmative diagnosis of rod-shaped nuclear virus (RV-PJ) infection in the kuruma shrimp (*Penaeus japonicus*), dark-field microscopy and transmission electron microscopy respectively were proposed by Momoyama *et al.* (1995). Electron microscopic examination had revealed the causative agent of white spot disease to be a rod-shaped, enveloped, non-occluded baculovirus (Inouye *et al.*, 1994; Momoyama *et al.*, 1994; Nakano *et al.*, 1994; Takahashi *et al.*, 1994; Chou *et al.*, 1995; Wang *et al.*, 1995). Complete white spot syndrome (WSS) virions and viral particles were observed in the epithelial cells of gills and stomach, foregut, lymphoid organ, heart, hepatopancreatic connective tissues, cuticular epidermis, hemolymph, reproductive organs and abdominal muscles of *P. monodon, P. vannamei, P. japonicus, P. stylirostris* and *Metapenaeus ensis* (Wongteerasupaya *et al.*, 1995a;

Inouye *et al.*, 1996; Murali Manohar *et al.*, 1996; Durand *et al.*, 1997; Lo *et al.*, 1997; Momoyama *et al.*, 1997; Wang *et al.*, 1997a, 1997b; Kasornchandra *et al.*, 1998). Lo *et al.* (1997) detected white spot baculovirus virus (WSBV) in the sub-cuticular epithelial cells of captured *Portunus sanguinolentus* and Supamattaya *et al.* (1998) observed the virus in the hypertrophied nuclei of pilaster cells, haemocytes and subcuticular epithelial cells of experimentally infected krill, *Acetes sp.* and crabs, *P. pelagicus* and *Scylla serrata*, using transmission electron microscope.

Early WSBV infection was indicated by nuclear hypertrophy and margination of chromatin (Wongteerasupaya *et al.*, 1995a). Eventhough, virions were confined mostly to the infected nuclei, some were seen either free in the cytoplasm or contained in vacuoles (singularly or in groups), where they are often associated with membranous structures (Durand *et al.*, 1997).

Complete virions were enveloped, ovoid to elliptical or rod-shaped and contained a partially lenticular shaped or cylindrical nucleocapsid with a highly electron dense core (Inouye *et al.*, 1996; Durand *et al.*, 1997; Momoyama *et al.*, 1997). In the negatively stained preparations, fully enveloped virions, nucleocapsids surrounded with envelope fragments and unenveloped capsids (free nucleocapsids) were observed (Durand *et al.*, 1996; Inouye *et al.*, 1996).

The sizes of the virions found in ultrathin sections were different among the viruses (PRDV, WSBV, SEMBV and HHNBV), and those, that had a negative stain were different among them (Takahashi *et al.*, 1998). According to Wongteerasupaya *et al.* (1995a), in *Penaeus monodon*, completely assembled virions measured 292 ± 29 nm by 111 ± 8 nm, while capsids measured 244 ± 28

nm by 80 ± 11 nm and nucleic acid cores measured 204 ± 28 nm by 66 ± 11 nm. But measurements, quite different from this were given by workers such as, Momoyama *et al.* (1995, 1997), Durand *et al.* (1996, 1997), Inouye *et al.* (1996), Sahul Hameed *et al.* (1998), Kasornchandra *et al.* (1998), Nunan *et al.* (1998), Supamattaya *et al.* (1998) and Rajendran *et al.* (1999). White spot virions, detected by Wang *et al.* (1997a, 1997b) in *Metapenaeus ensis* (221 ± 6 nm by 107 ± 7 nm) were smaller than those found in *P. monodon* (298 ± 21 nm by 107 ± 8 nm) and in *P. japonicus* (248 ± 12 nm by 104 ± 8 nm). WSSV particles, similar in shape and size (245-290 nm by 120-125 nm) to that detected in WSSV infected shrimps were observed in experimentally challenged krill, *Acetes sp.* and crabs, *Portunus pelagicus* and *Scylla serrata* by Supamattaya *et al.* (1998). The nucleocapsids were loosely surrounded by a trilaminar envelope of 6-7 nm thickness (Durand *et al.*, 1997), which consisted of two electron opaque layers, separated by one electron lucent layer (Inouye *et al.*, 1996; Kasornchandra *et al.*, 1998).

In negatively stained preparations, the cylinder representing the nucleocapsid was closed at one extremity by a smaller segment that forms a slightly rounded or pointed end, while the opposite extremity was squared or flat (Durand *et al.*, 1997; Sahul Hameed *et al.*, 1998). The 'extended' nucleocapsids displayed a superficially segmented appearance with electron opaque bands (17-18 nm), alternating with electron lucent bands (3-5.5 nm), arranged perpendicular to the long axis of the nucleocapsid in a stacked series (Wongteerasupaya *et al.*, 1995a; Durand *et al.*, 1996, 1997; Sahul Hameed *et al.*, 1998). Degraded nucleocapsids, with cross-hatched appearance and a clear area (24 nm thick) at one extremity were observed and were fractured at the level of segmentation. The content of the capsid seemed to be a fine, electron dense, central, tenuous

filamentous material, released during degradation of the nucleocapsid. Different particles with a size of 400 nm (300-460 nm) by 120 nm (100-150 nm) were also observed, which appeared larger than the nucleocapsids and the virions.

In *Penaeus monodon* specimens of twelve hour post injection with the viral extract, signs of viral replication were observed (Wongteerasupaya *et al.*, 1995a). Durand *et al.* (1997) studied the morphogenesis of WSBV in detail. In some hypertrophied nuclei, capsid originators (Johnson, 1988), measuring 60 nm in diameter (Inouye *et al.*, 1996), immature viruses with empty capsids, nucleocapsids and circular envelopes were observed. This indicated virus multiplication and assembly in the nucleoplasm and occluding protein (polyhedrin) was absent (Wongteerasupaya *et al.*, 1995a; Momoyama *et al.*, 1997; Wang *et al.*, 1997a, 1997b). In their opinion, the capsid was formed from a 'capsid originator' and the membrane was formed 'de novo' in the nucleoplasm.

Certain tubular structures (nucleocapsid precursors), having diameters close to those of empty capsids and segmentation corresponding to that of nucleocapsids were seen in the infected nuclei of cells in WSS affected penaeid prawns (Durand *et al.*, 1997). The space between the envelope and the capsid was reduced and the open end was large. Some partially enveloped capsids were also noted (Inouye *et al.*, 1996). In highly infected nuclei, forming virions were located centrally, while mature virions tended to be concentrated along the nuclear margin. Mature virions were assembled in ordered paracrystalline arrays or randomly scattered inside the nuclei (Durand *et al.*, 1997; Kasornchandra *et al.*, 1998; Rajendran *et al.*, 1998). Lo *et al.* (1997) observed viral particles both in the nucleus and cytoplasm of the infected follicle cells and the connective

tissue layer surrounding the seminiferous tubules of the testis of captured *P. monodon* brooders.

However, there has been no published work on WSBV in *Penaeus indicus* at the ultrastructural level. As *P. indicus* is a very much favourable species for coastal aquaculture in India, especially in Kerala, the present investigation will give detailed information on the ultrastructural changes in the various target organs of WSBV, such as gills, compound eye, heart, cuticular epidermis and foregut in *P. indicus*. A thorough knowledge of the ultrastructural details of the etiological agent is a must in order to purify and isolate the virus and for further advanced works like development of gene probes, vaccines etc. An attempt has been made in the present study to reveal the various stages of viral development and release in the target tissues studied.

3.2 MATERIALS AND METHODS

Live specimens of *Penaeus indicus*, which clearly exhibited the symptoms of white spot syndrome (WSS), as described in the chapter 2, were selected for ultrastructural studies. Tissues, such as cuticular epithelium, gills, foregut, heart and eye from the morbid specimens were the main targets of present investigation and were processed as follows: initially, the gills were swabbed with fresh, cold (4 °C) fixative (3%, buffered gluteraldehyde solution) on a piece of sterile cotton. Three injections of the fixative, 0.5 ml each were given using a sterile, 1ml syringe and 21 gauge needle; first two at the top and bottom of the hepatopancreatic region and a third at the junction of fifth and sixth abdominal segments, by inserting the needle gently into the musculature, just below the cuticle. Immediately, the selected tissues were dissected out and immersed in freshly prepared, buffered gluteraldehyde (3 %, 0.1 M

gluteraldehyde solution, buffered to pH 7.3 with 0.1 M sodium cacodylate), taken in separate, labeled vials, for two hours at 4 °C. The tissues were washed three times in 0.1 M cacodylate buffer, to remove the excess aldehydes, for about half an hour duration each, with frequent shakings at intervals for uniform penetration of the buffer. The tissues were then trimmed into appropriate size (1 x 1 mm), with a razor blade. The tissues were then post-fixed in freshly prepared 1% osmium tetroxide solution in double distilled water, for two hours at 4 °C. Proper post-fixation was indicated by the blackening of the tissues and they were washed in 0.1 M cacodylate buffer, giving three changes of fifteen minutes duration each. The samples were left overnight in fresh buffer solution at 4 °C.

After decanting the buffer completely, the tissues were dehydrated through graded acetone series (30 %, 50 %, 70 %, 90 %, 100 %) for a duration of 45 minutes in each grade, at 4 °C with a final washing in absolute acetone for 30 minutes. After dehydration, the samples were infiltrated with liquid resin (spurr) in separate vials for one hour each (Spurr, 1969).

Infiltration was followed by embedding. The composition of the freshly prepared embedding medium was ERL –7.5 g, DER – 4.5 g, NSA –19.5 g and Dimethyl Amino Ethanol (DMAE – 0.3 g). The ingredients were mixed well and poured into small embedding capsules. The processed tissues were transferred to different capsules and allowed to settle by themselves at the pointed ends of the capsules. Four replicates were prepared for each tissue. The samples were labeled properly by inserting small pieces of paper, having the details, marked with pencil, into the embedding medium in the capsules. The capsules, containing the tissues and resin were allowed to remain at room temperature for one hour and then incubated at 60 °C for 4 hours and later temperature is

increased to 70 °C and kept for 8 to12 hours to allow complete polymerization of the resin.

Semi thin sections of 500 nm thickness were taken with glass knives using an ultramicrotome (Leica Ultracut R), stained with toluidine blue and observed under a light microscope, in order to identify the affected region. These blocks were again trimmed for ultramicrotomy and ultra thin sections (50-60 nm) were taken by using freshly cut glass-knife. The ribbon of ultra thin sections were allowed to float on distilled water taken in a plastic-boat shaped trough, attached to the glass-knife with molten paraffin wax.

Nickel grids (300 mesh size, 3.0 mm diameter, Ladd, USA) were carefully introduced under the ultra thin sections, floated in the water, with the dull side of the grids facing the sections. The ribbon of sections on the grids were gently lifted up and dried on filter paper for a few minutes. Then, they were stained with filtered uranyl acetate solution (2 g uranyl acetate in 10 ml of 30 % ethanol) for 20 minutes and rinsed thrice in double-distilled water. The sections were further stained with freshly prepared, filtered 0.42 % lead citrate solution (Reynolds, 1963) for eight minutes and dipped thrice in double-distilled water. They were dried well and screened under a transmission electron microscope (HITACHI H-600 A), observed at 50 KV. Required ultrastructural details were examined, studied and photographed on ORWO-125 ASA film. The length and width of the viral particles were also measured.

3.3 RESULTS

SUBCUTICULAR EPITHELIUM

The subcuticular epithelium covering the general body surface and appendages of penaeid prawns affected with WSD was one of the major targets of WSBV. The virus infection and multiplication of the virions were clearly observed in the subcuticular epithelial cells of *Penaeus indicus*. Uninfected cells were also present along with the infected cells. The nuclei of these cells had a spherical nucleoli s, intact nuclear membrane and well developed euchromatin and heterochromatin. Cell membranes and cytoplasmic organelles were also visible. But with the viral infection, the nuclei became hypertrophied and their shape also changed. The entire cell area was occupied by the hypertrophied nucleus and the cytoplasm was pressed against the cytoplasmic membrane, which formed a narrow rim around the infected nucleus. Margination of the chromatin along the nuclear membranes in the distorted nuclei was a common feature of the viral infection (Fig. 38 A).

Morphogenesis and assembly of the viral particles in the infected nuclei were well observed (Figs. 38 B & 38 C). Empty capsids, cylindrical nucleocapsids and virions with envelopes were seen in these figures. Long and short tubular structures and numerous circular and linear membranous fragments were also present. Fully and partially enveloped virions were observed. The virions were bacilliform in shape and the nucleic acid core was highly electron-dense. The virions, nucleocapsid and the empty capsids measured 250-325 nm x 85-120 nm, 180-254 nm x 75-82 nm and 150-250 x 75-110 nm respectively. One end of the nucleocapsid was pointed and the other was slightly blunt in some

virions, while both the ends were pointed in others. A small bulging of the envelope could be seen on one side in some virions. A nipple like envelope extension at one of the pointed ends was also observed in a few virions. Apart from the envelope extension, at one end, the opposite extremity also exhibited slight protrusion of the envelope in some virions (Fig. 38C). Empty capsids, without an envelope could be observed in this figure. The envelope had a trilaminar structure and the area between the nucleocapsid and the envelope was electron lucent. In some virions, this space was very broad, ranging from 8 to 9 nm, whereas, in others, this was very narrow (6-7 nm). Even in a single virion, the area between the nucleocapsid core and the envelope was not uniform throughout (Fig. 38C). Small, round, electron-dense, structures were also seen between the developing viruses, which measured about 31-50 nm in diameter.

The mature virions, get arranged themselves in paracrystalline arrays at the periphery of the nucleus. In fig. 38D, the virions are arranged in small groups. A random distribution of the virions could be observed (Fig. 38E). Sometimes the entire nucleus was occupied by virions (Fig. 38F). After attaining maturity, the virions seemed to emerge through the broken nuclear membrane and present in the cytoplasm. Groups of virions were seen dispersed in the cytoplasm. They were immersed in a granular mass in the cytoplasm. They were also distributed singularly in the cytoplasm or associated with vacuoles. Long tubules, measuring 600 to 720 nm x 80-100 nm size were also observed among the forming viral particles (Fig. 38B).

Fig. 38. Transmission electron micrographs of cuticular epithelium of *Penaeus indicus* affected with white spot disease

A Early stage of infection in the nucleus of a cuticular epithelial cell (mc-marginated chromatin; rer-rough endoplasmic reticulum) x 8000.

B Hypertrophied nucleus containing virions in various stages of assembly in a cuticular epithelial cell (v-virion; t-tubular structure) x 10,000.

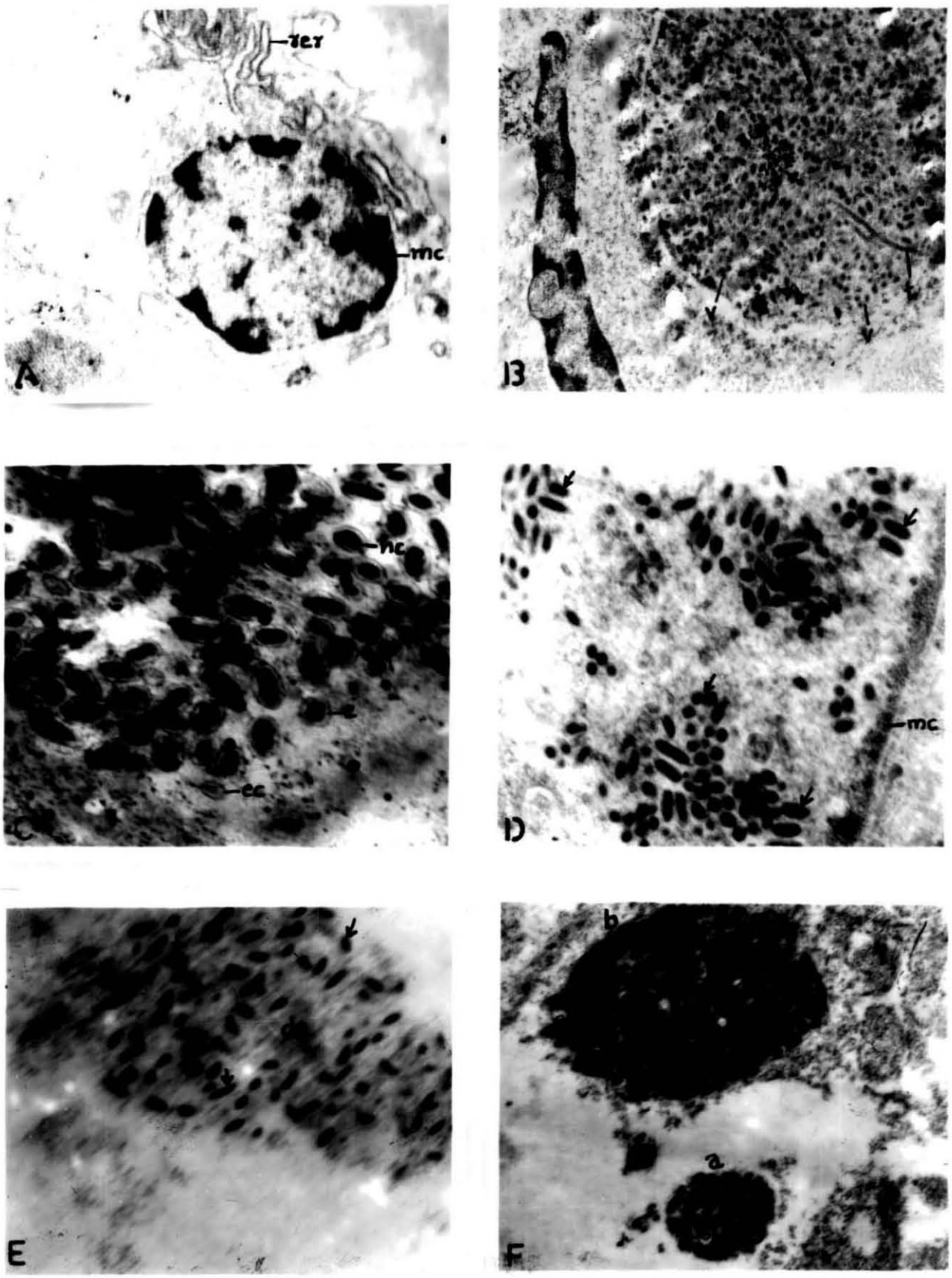
C Enlarged view of enveloped virions in the affected nucleus (ec-empty capsid; e-envelope; nc-nucleocapsid) x 40,000.

D Ultrathin sections showing white spot disease virions arranged in paracrystalline arrays (mc-marginated chromatin; arrows indicate virions) x 25,000.

E Maturing viral particles seen along with disintegrated chromatin (dc) in the infected nucleus. Arrows indicate viral particles. x 25,000.

F Nuclei (a & b) of adjacent cuticular epithelial cells filled with virions. x 25,000.

FIGURE 38



GILLS

Epithelial cells of the gill appeared one of the most common target organs of WSBV. Apparently normal, unaffected, epithelial cells were also observed along with the affected cells. The cell membranes were intact. In such cells, cytoplasmic organelles such as endoplasmic reticulum, ribosomes, mitochondria etc. were present. A well defined nucleus with nuclear membrane, almost centrally located nucleolus and euchromatin could be clearly distinguished in these cells. The nuclei had a definite size and shape. The nuclei of the adjacent cells showed only little variability in size and shape (Figs. 39A &39B). Adjacent epithelial cells were clearly distinguished. Rough endoplasmic reticulum were seen in arrays around the nuclei, in continuation with the nuclear membranes.

As a result of the infection by WSBV, both the structural and functional integrity of the gill tissue had been lost. The infection was indicated by a marked change in the shape and size of the nucleus. Nuclei became almost circular or oval in shape as the process of hypertrophy began. Nucleoli disappeared and the chromatin became marginated and disintegrated (Fig. 39C). Small fibrillar structures appeared in the nucleoplasm, leading to the formation of virogenic stroma. Infected haemocytes also showed similar structural alterations. Some of the infected epithelial cells still retained the cell membrane and nuclear membrane (Fig. 39D). But the cytoplasm and the nucleoplasm became granular, without having any structurally intact organelles.

Viral development and their assembly in the infected nucleus of the epithelial cell were observed in the present study (Figs. 39E & 40A). Viral

Fig. 39. Transmission electron micrographs of gill epithelium of *Penaeus indicus* infected with WSDV.

A Ultra thin sections of gill epithelium without WSS virions (n-nucleus; nm-nuclear membrane; nu-nucleolus; rer-rough endoplasmic reticulum; hc-heterochromatin; ec-euchromatin) x 10,000.

B Nuclei of infected and non infected gill epithelial cells (n-non infected nucleus; in-infected nucleus with virions inside; arrows indicate virions) x 8,000.

C WSD affected nucleus showing disintegrated chromatin (dc). Note the disappearance of nucleolus and nuclear membrane. x 10,000.

D Ultrathin section of the infected nucleus of a gill epithelial cell (t-tubular structures; nm-nuclear membrane; cm-cell membrane; dct-disintegrated cytoplasm; arrows indicate viral particles) x 17,000.

E Hypertrophied nucleus of a gill epithelial cell showing intranuclear viral particles (mc-marginated chromatin; arrows indicate viral particles) x 20,000.

F A part of the hypertrophied nucleus of gill epithelial cell showing viral particles (arrows), immersed in virogenic stroma (vs); ec-empty capsid. x 17,000.

FIGURE 39

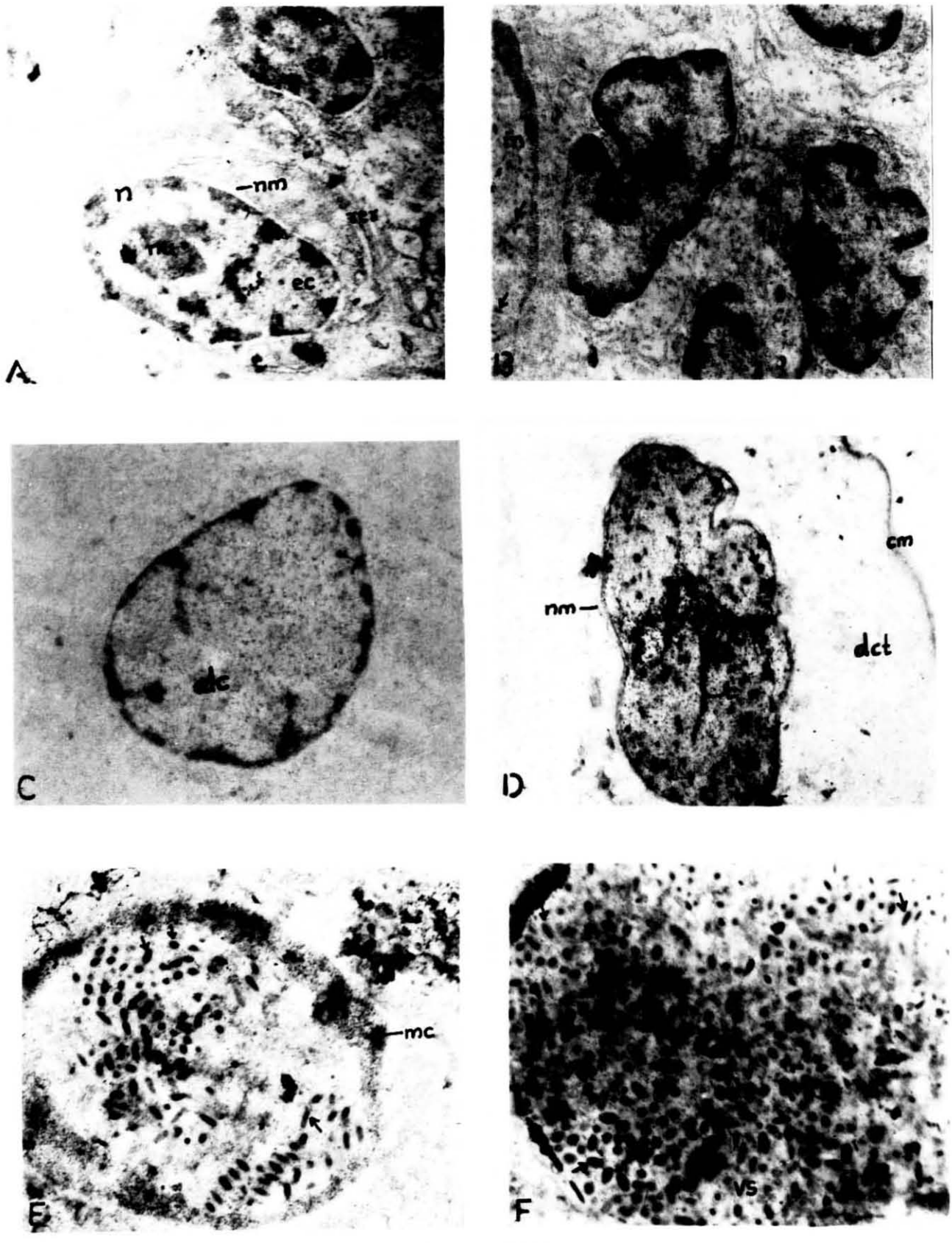


Fig. 40. Transmission electron micrographs of gill epithelium of *Penaeus indicus* affected with WSD showing viral particles in the infected cells

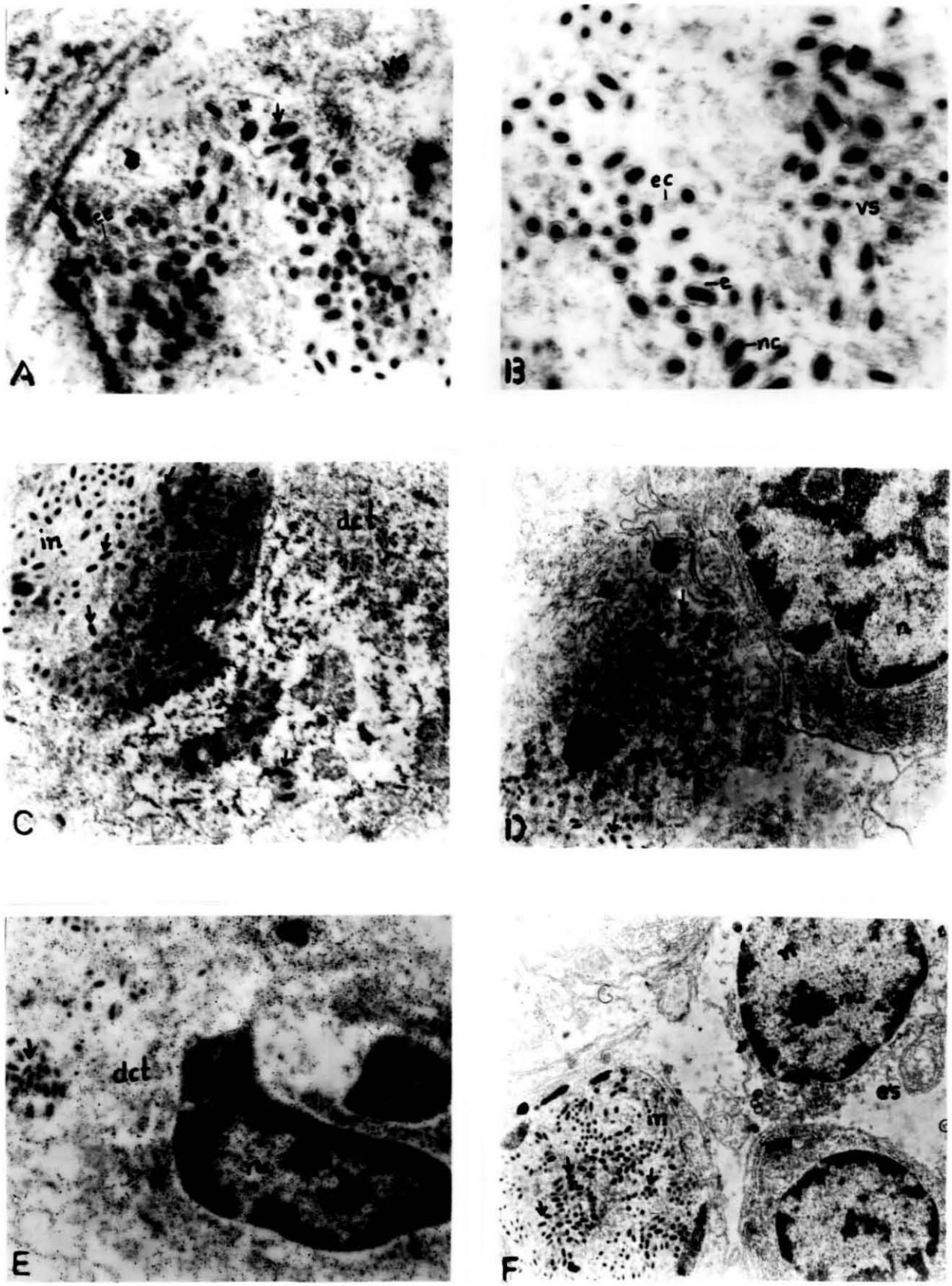
A A portion of the hypertrophied gill epithelial cell showing viral particles (vs-virogenic stroma; arrows indicate viral particles) x 20,000.

B An enlarged view of bacilliform virions in the hypertrophied nucleus of gill epithelial cell (e-envelope; nc- nucleocapsid; ec-empty capsid; vs-virogenic stroma) x 30,000.

C, D & E Ultrathin sections of infected gill epithelial cells showing groups of virions dispersed in the cytoplasm (n-nucleus of non infected cell; in-infected nucleus; rer-rough endoplasmic reticulum; nu-nucleolus; dct-disintegrated cytoplasm; arrows indicate virions) x 15,000 (C), x 10,000 (D), x 12,000 (E).

F An area of the gill epithelium showing both infected (in) and non infected nuclei (n) (nu-nucleolus; rer-rough endoplasmic reticulum; es-empty space; arrows indicate virions in the infected nucleus) x 8,000.

FIGURE 40



particles in the various stages of development, such as empty capsids, partly and fully enveloped virions, tubular and circular membranous structures etc. were seen in the central part of the infected nuclei. Virions were ovoid to rod shaped with pointed or blunt ends. Enveloped, mature virions measured about 230-310 nm x 90-115 nm and cylindrical nucleocapsids about 178-246 nm x 70-78 nm in size. 235 ± 15 nm x 113 nm sized enveloped empty capsids and those without an envelope were also observed. The nucleocapsids were loosely surrounded by a trilaminar envelope of 6-9 nm thick (Fig. 40B). An envelope extension was also visible in some virions. Fully formed virions had a nucleic acid core, more electron dense than that in the other virions. The characteristic paracrystalline arrangement of the mature virions was seen along the periphery of the infected nuclei (Fig. 39E). In fig. 39F, the entire area of the nucleus was found to be occupied by the virions, without leaving the remnants of the nuclear inclusions.

The infected nuclei usually had a faint, cloudy boundary, which was also incomplete at several places. The virions seemed to travel in small groups to the adjacent cells for fresh infection, causing the disintegration of the cytoplasm and its membranous structures (Figs. 40C, 40D & 40E). In some cells, large vacuoles were seen, replacing the cytoplasmic organelles. These vacuoles sometimes contained virions, either singularly or in groups. A comparison of the uninfected and infected nuclei could be seen in fig. 40F.

STOMACH

The subcuticular epithelial cells of the stomach was one of the most commonly affected tissue of the penaeid prawns by WSBV. The viral particles were observed in this tissue, even in the initial stages of infection and stomach

epithelium was one of the indicator tissues of the viral infection. Apparently normal and uninfected nuclei of the stomach epithelial cells of *Penaeus indicus* had the same common features, such as an intact nuclear membrane, diffused chromatin and an almost centrally located, round nucleolus. The nucleus had more or less definite size and shape. Cytoplasmic organelles and cell boundaries could be sharply distinguished. Rough endoplasmic reticulum appeared intact in structure.

In WSBV-infected stomach epithelial cells cellular structure was altered completely. Cytoplasmic organelles were severely damaged, leaving large spaces, filled with fine granular materials. Their nuclei also exhibited pronounced abnormalities including disappearance of the nucleoli and nuclear membrane. The chromatin became marginated and less electron-dense. The central areas of the infected nuclei contained fine granular and fibrillar materials, indicating viral replication and assembly. The shape and size of the nucleus also showed marked changes as seen in fig. 41A. The nucleus became hypertrophied. Figs. 41B and 41C gave a comparison of the fully infected nuclei and the neighbouring apparently healthy and uninfected nuclei. Another notable feature is the dilatation of the rough endoplasmic reticulum, before disintegrating into small fragments (Fig. 41D). Empty capsids, nucleocapsids, virions, envelope fractions, tubular and circular structures could be observed in the infected nuclei (Fig. 41E). The nucleic acid core was highly electron dense in matured virions.

The enveloped virions were rod-shaped with pointed or blunt ends. Enveloped virions, nucleocapsids and empty capsids, measured 245-326 nm x 80-120 nm, 216 ± 36 nm x 67-74 nm and 200 x 104 ± 4 nm respectively. Though, most of the virions appeared to have both the ends pointed, there was a slight

Fig. 41. Transmission electron micrographs of stomach epithelial cells of *Penaeus indicus* affected with white spot disease

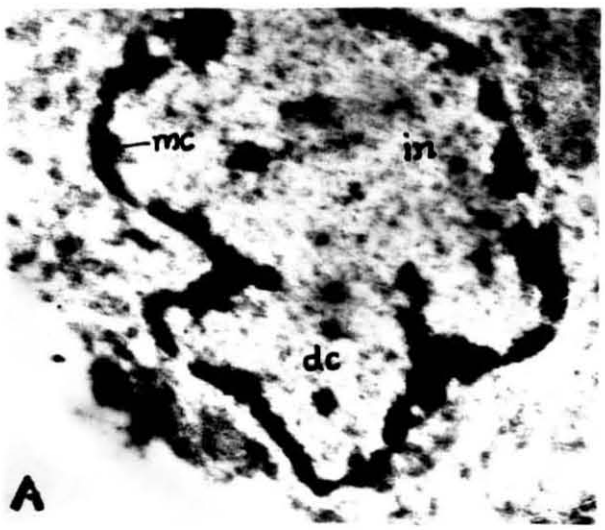
A A nucleus in the initial stage of infection (in-infected nucleus; mc-marginated chromatin; dc-disintegrated chromatin; note the disappearance of nucleolus) x 8,000.

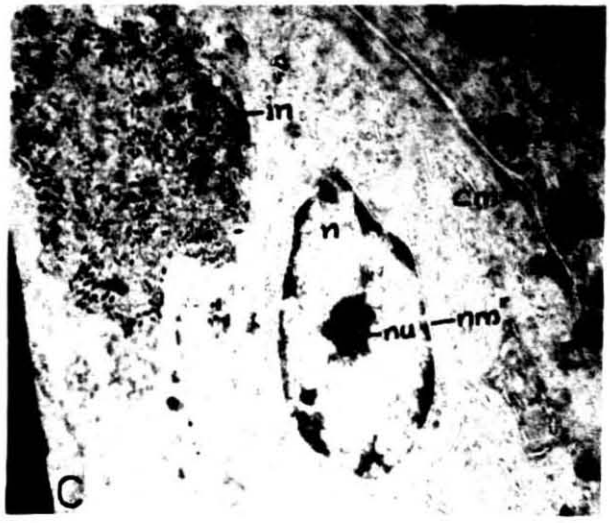
B & C Adjacent cells of foregut epithelium showing both non infected (n) and infected hypertrophied nucleus(in) (nu-nucleolus; nm-nuclear membrane; cm-cell membrane) x 3,500.

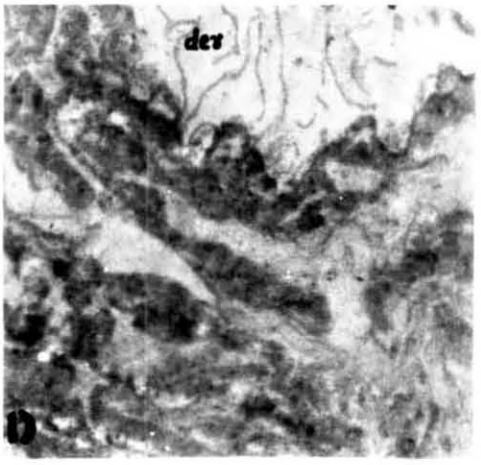
D Cytoplasmic disintegration in the infected cells (der- dilated endoplasmic reticulum) x 8,000.

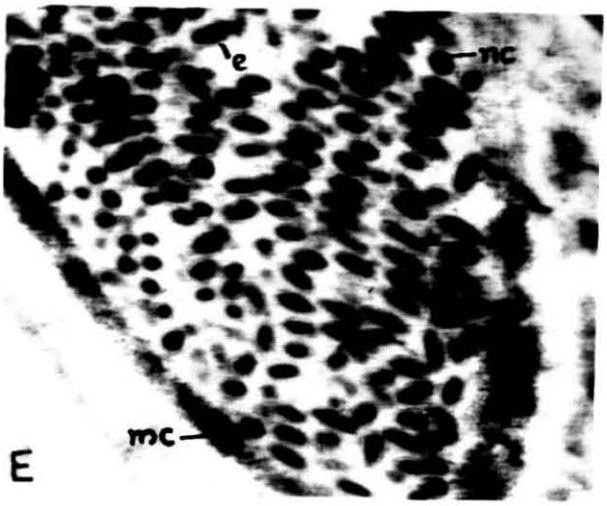
E Paracrystalline arrangement of virions in the hypertrophied nucleus before release (nc-nucleocapsid; e-envelope; mc-marginated chromatin) x 30,000.

FIGURE 41









variation in the shape of the two extremities in some virions. In them, one end is pointed and the other is slightly flat or blunt. The nucleocapsid core was highly electron dense. The envelope was trilaminar, made up of two electron-dense layers, separated by an electron-transparent layer. The area between the nucleocapsid and the envelope was 6-9 nm. A minute projection of the envelope was seen on the pointed end in some virions. Both the C.S. and L.S. of the virions could be visible in the ultrathin sections of the infected nuclei (Fig. 42A).

Some of the infected nuclei became greatly enlarged and they occupied the entire cell as in fig. 42B. They were completely filled with the virions and in certain incidences, no remnants of the nuclear inclusions were retained. The concentration of the virions in the infected nuclei was observed to be very high in the foregut epithelial cells (Fig. 42D).

A very characteristic feature of the WSBV infected nuclei of the foregut epithelial cells was the orderly, paracrystalline or honeycomb-like arrangement of the matured virions along the periphery of the hypertrophied nuclei (Fig. 42C). This particular, orderly arrangement of the virions could be seen throughout the nucleus in fig. 41B. The immature viruses and the granular matrix were seen at the centre of the infected nuclei. Sometimes, they were randomly distributed. Remnants of the nuclear membrane were still retained in them. In fig. 42D, fragment of the nuclear membrane was present on one side of the nucleus, which was completely filled with the virions, whereas, it was completely absent on the other side.

After attaining maturity in the nucleus, complete, enveloped virions were released by the breakage of the nuclear membrane or through the already

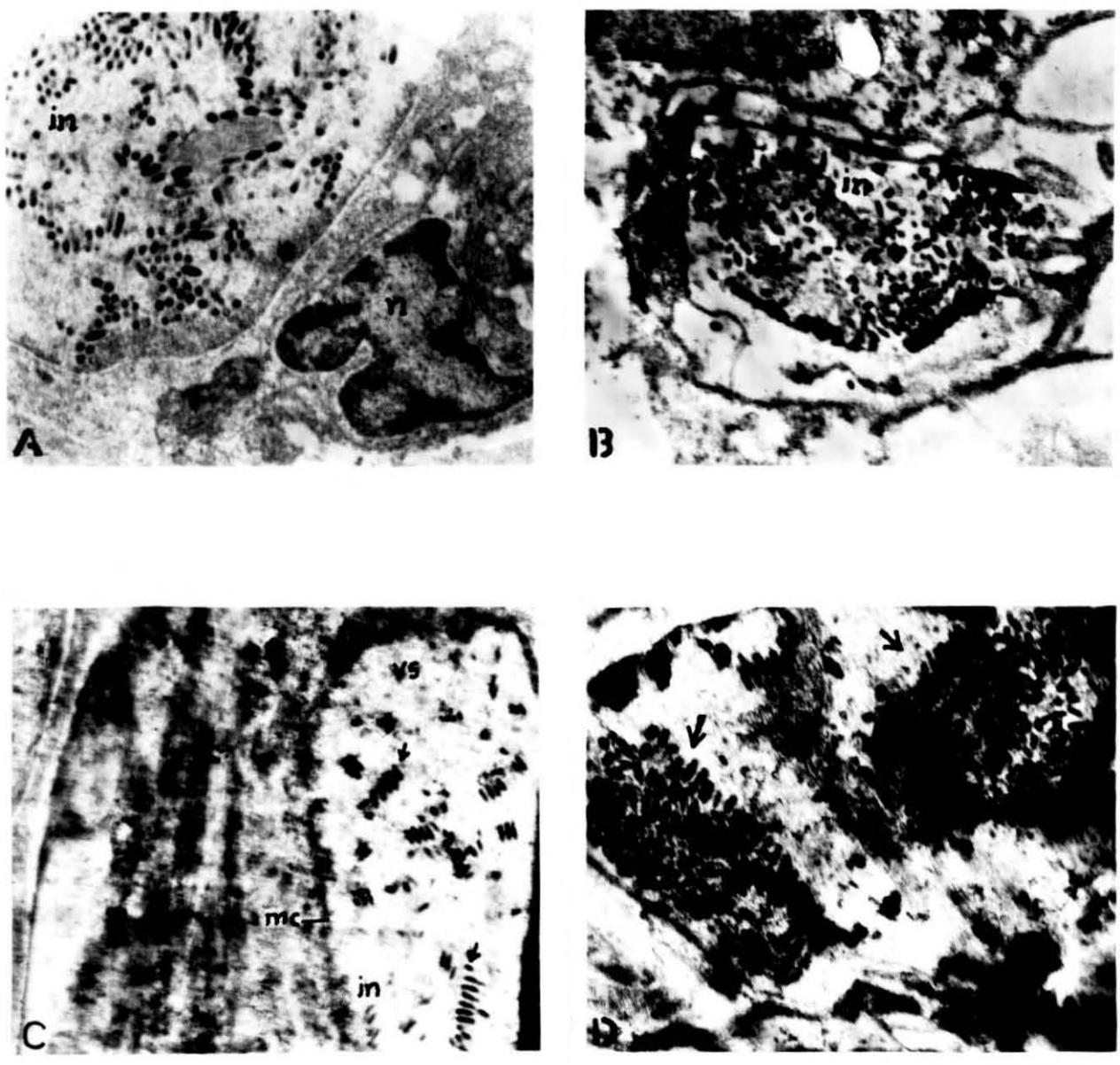
Fig. 42. Transmission electron micrographs of stomach epithelium showing the presence of viral particles in the infected cells

A & B Ultrathin sections of stomach epithelium showing viral particles in various stages of development in the hypertrophied nuclei of the infected cells (in-infected nucleus; n-non infected nucleus) x 15,000 (A), x 12,000 (B).

C Beginning of the peculiar paracrystalline arrangement of virions indicated by arrows in the infected nucleus (in) (vs-virogenic stroma; mc-marginated chromatin) x 12,000.

D The entire infected nucleus fully packed with virions. x 17,000.

FIGURE 42



broken nuclear membrane. They moved in-groups to the nearby fresh cells (Fig. 41E). Vast areas of the infected tissue had a disintegrated multinucleated appearance. Sometimes, groups of virions or singular ones were observed to be associated with vacuoles. Mature virions were often seen in the cytoplasm also. Around them, the cytoplasmic organelles were seen disintegrated into fibrillar or fine granular materials. Large voids were also present due to the lysis of the cellular materials.

HEART

Heart tissue formed one of the major target organs of WSBV infection in penaeid prawns. The virus most frequently affected nuclei of the myocardial cells and the connective tissue cells in the heart. The tissue as a whole was damaged severely. Viral particles were observed in the hypertrophied nuclei of the haemocytes and cardiac cells. Along with the infected nuclei, few numbers of apparently normal nuclei were also present. The seemingly uninfected nuclei had intact nuclear membrane, and diffused chromatin, which was highly electron-dense. A well-defined, almost round nucleolus was also present in the centre of the nucleus. These nuclei had a definite size and shape and were easily distinguished from the infected ones. Arrays of rough endoplasmic reticulum were visible around the nucleus, associated with the nuclear membrane (Figs. 43A & 43B). Cell boundaries and cytoplasmic organelles were quite distinct. Striated muscle fibres were present in bundles, with small nuclei in the centre of each bundle.

Earlier stages of infection were indicated by marked nuclear hypertrophy (Fig. 43C). The nuclear membrane and nucleolus disappeared and

the diffused chromatin became less electron dense and marginated. Fig. 43D shows the condition of the infected nuclei, just before the viral development and assembly. The entire structure of the nucleus was lost and its contents had been disintegrated into a fine granular mass. Fine fibrillar structures were also present, forming the virogenic stroma. The gradual disintegration of the nuclear materials could be seen. In fig. 43C, in one of the two nuclei, the nuclear membrane and chromatin were present on one side, the opposite side having been disintegrated completely.

The cytoplasm around the infected nuclei also revealed marked abnormalities. The cytoplasmic organelles disintegrated into small fragments and the cells lost their boundaries. In fig. 43C, the remnants of the endoplasmic reticulum were observed. But in some infected cells, the configuration of the cytoplasm was completely lost, without even leaving the remnants of the organelles.

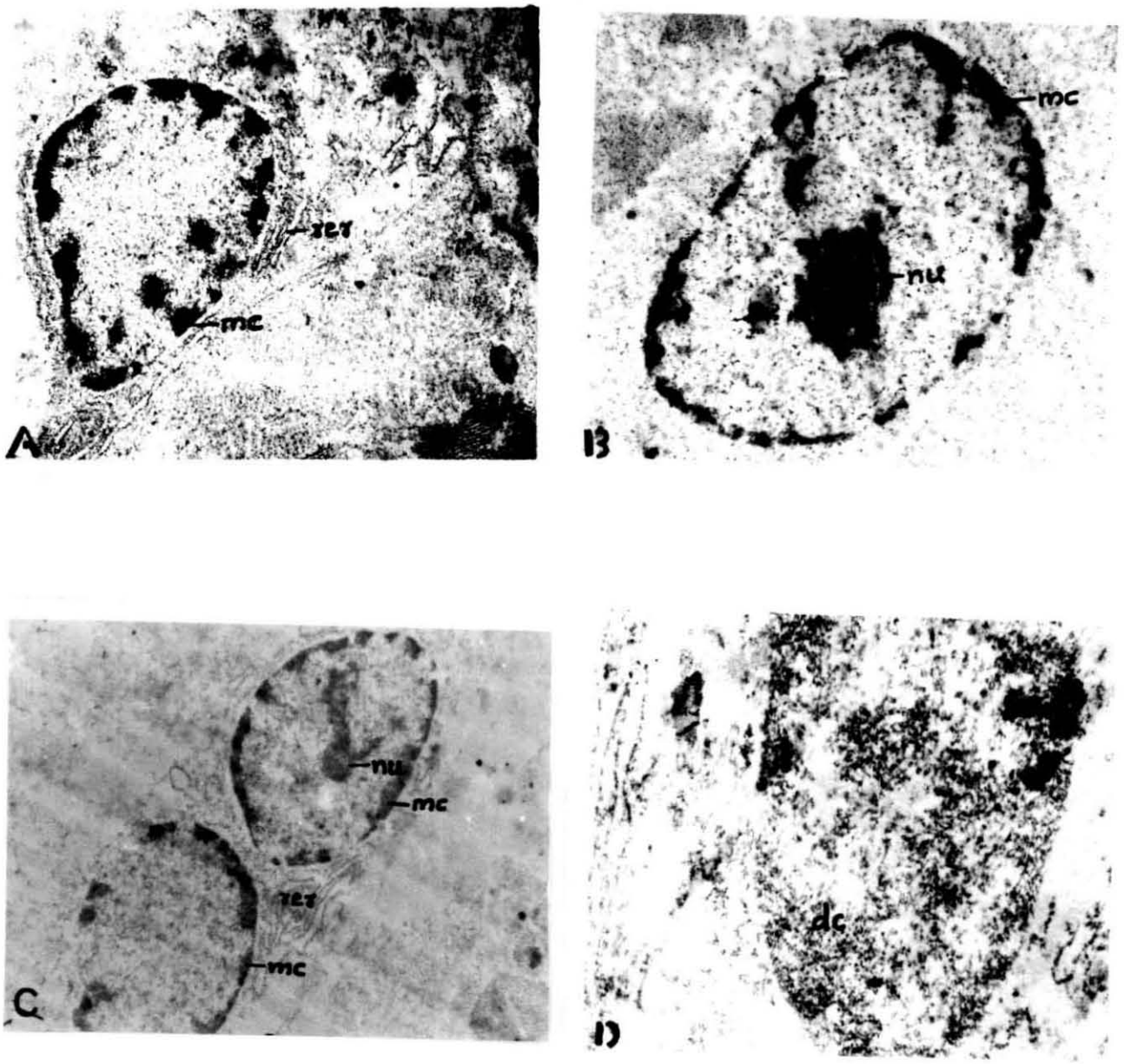
Soon after the disintegration of the nuclear materials, morphogenesis of the viral particles and their assembly began. Figs. 44A and 44B showed the various stages of viral assembly. Immature viruses with empty capsids, nucleocapsids and circular structures were seen in the above figures. A large number of circular and linear membrane fragments were observed centrally. Empty capsids measuring about 200 nm x 117 nm and long tubules of size, 530-670 nm x 108 ± 10 nm were found along with fibrillar and granular materials. Ovoid to rod-shaped, complete virions, surrounded by trilaminar envelopes were seen, mostly along the periphery of the infected nuclei. The matured, enveloped virions measured 240-282 nm x 100 ± 20 nm and the nucleocapsids alone measured 180-250 nm x 70-75 nm. Both the ends were pointed in some, whereas

Fig. 43. Transmission electron micrographs of myocardial cells of *Penaeus indicus* infected with WSDV

A, B & C Ultrathin sections of the nuclei of myocardial cells showing early stages of infection (rer-rough endoplasmic reticulum; nu-nucleolus; mc-marginated chromatin) x 9,000(A), x 10,000 (B), x 8,000 (C).

D Nucleus of myocardial cell showing disintegrated chromatin (dc) x 15,000.

FIGURE 43



in others, one end was pointed and the other end was slightly blunt. The nucleic acid core was highly electron-dense, compared to the envelope (Fig. 44A).

The envelope itself was made of two electron-dense layers, separated by an electron-lucent layer in between. The envelope varied in shape from oval to round. A very small, nipple-like extension of the envelope was seen at one of the extremities in some virions. The electron-lucent area between the envelope and the nucleocapsid was 6-8 nm wide. Partially and fully enveloped capsids were also seen.

In some infected nuclei, as in figures, 43C and 44B, the remnants of the disintegrated chromatin were seen along the periphery of the nuclei. Mature virions, with the envelope were observed to emerge from the nucleus through the broken nuclear membrane. These complete, virions seemed to infect the nearby fresh nuclei. The released virions moved in different directions, through the disintegrated cytoplasm, in small groups to the fresh, adjacent cells and entered into their nuclei for further infection and multiplication. In figs. 44C, 44D and 44E, a few virions were seen near normal nucleus. The area around the virions was vacant without any cytoplasmic organelles. Disintegrated, granular material was present in between the virions. Damaged mitochondria were seen in figs. 44C and 44D. The mitochondria lost the entire structure, except, for the boundaries. Rough endoplasmic reticulum, seen near the nucleus also started disintegrating. The number of virions, approaching a particular nucleus for infection seemed to be very few compared to those released from the nucleus, after their development and replication in that particular nucleus. In some cases, the nucleus was completely occupied by the virions, so that not even a single

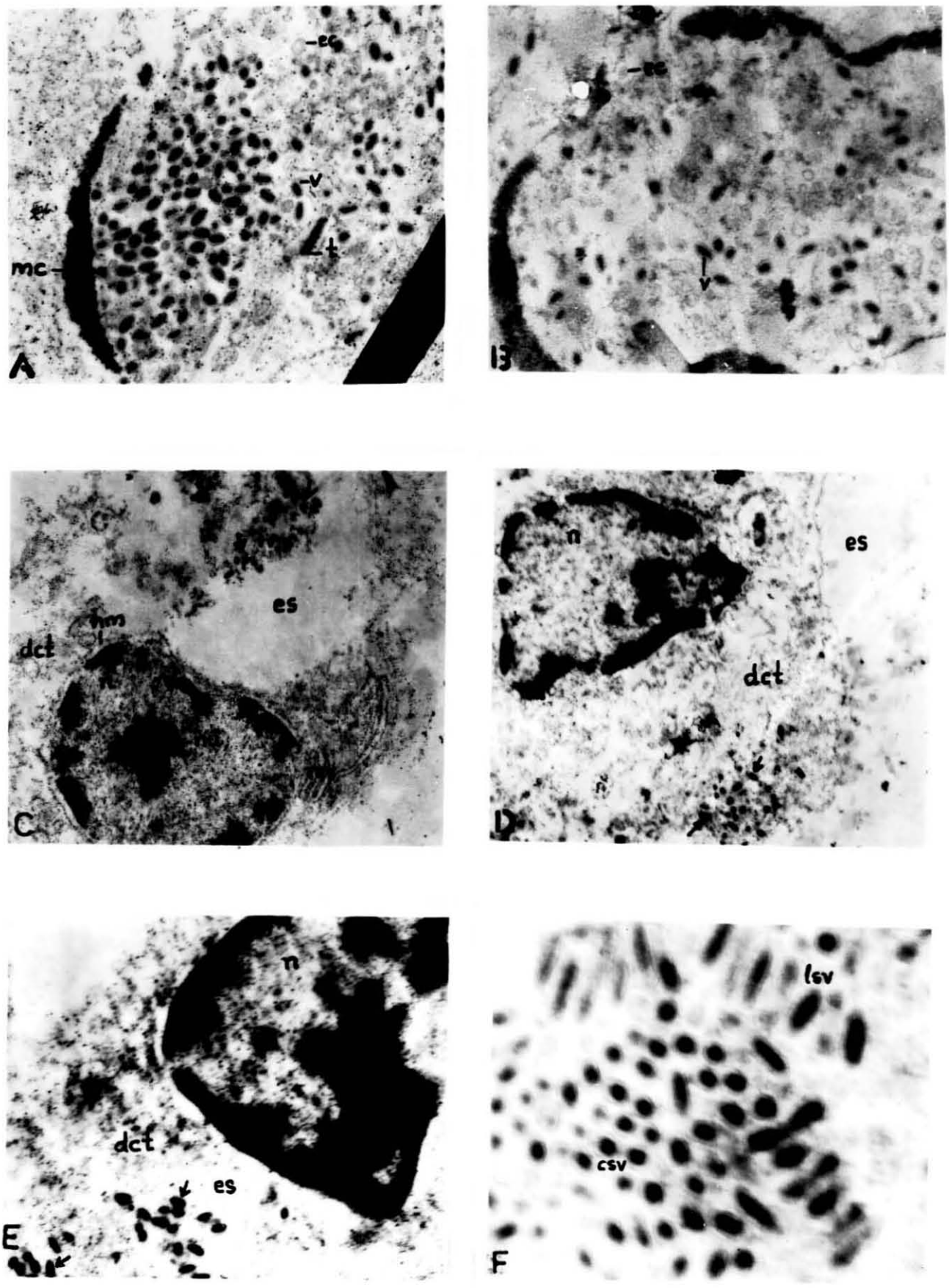
Fig. 44. Transmission electron micrographs showing viral particles in the myocardial cells of *Penaeus indicus* affected with WSD.

A & B Hypertrophied nucleus of myocardial cell with viral particles in various stages of development (v-virion; t-tubular structure; ec-empty capsid; mc-marginated chromatin) x 15,000 (A), x 25,000 (B).

C, D & E Ultrathin sections of virions dispersed in the cytoplasm due to disintegration of the infected nucleus (n-non infected nucleus; nu-nuleolus; nm-nuclear membrane; rer-rough endoplasmic reticulum; es-empty space; dct-disintegrated cytoplasm; arrows indicate virions) x 8,000 (C), x 10,000 (D), x 20,000 (E).

F Ultrathin sections of virions showing paracrystalline arrangement along the periphery of the hypertrophied nucleus (lsv-L.S. of virion; csv-C.S. of virion) x 50,000.

FIGURE 44



fragment of the nuclear inclusion was left behind. The virions were arranged around the periphery of the nucleus to form a paracrystalline pattern (Fig. 44F).

Retinular cells of the compound eye formed one of the major target areas of WSBV infection. Multinucleated cells were also present in this area (Fig. 45A). Developing and maturing virions were seen along with the granular virogenic stroma in the hypertrophied nuclei of the retinular cells (Figs. 45B & 45C). Both the non infected and hypertrophied infected nuclei were found nearby. Cell boundaries and cytoplasmic organelles were not present in it. Oval to rod-shaped enveloped virions and certain tubular and circular membranous structures were also observed in one of the affected nuclei. Nucleolus was absent and margination of the chromatin was very clear. Fig. 45D showed the specific paracrystalline or honeycomb like arrangement pattern of the matured virions along the periphery of the infected, markedly hypertrophied nucleus of the retinular cell of the compound eye. In it, matured virions were arranged in small patches. Figs. 45E and 45F give and enlarged view of the matured virions, having highly electron dense nucleic acid core and a trilaminar envelope.

Fig. 45. Transmission electron micrographs of the retinular cell nuclei region of the compound eye of *Penaeus indicus* affected with WSD

A A retinular cell showing early stages of WSDV infection (n-nucleus; in-infected hypertrophied nucleus with disintegrated chromatin) x 15,000

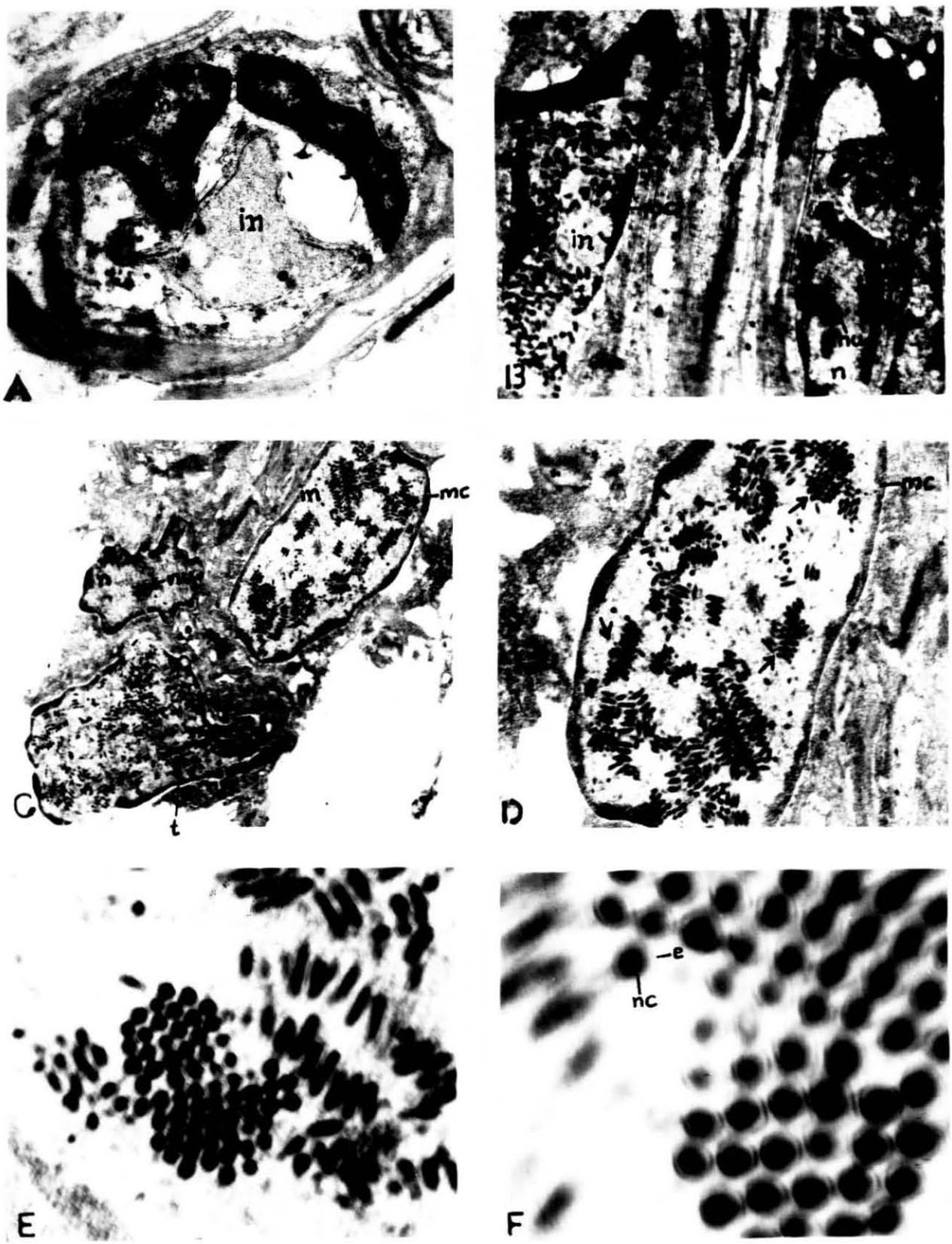
B & C A comparison of the non infected nucleus(n) and hypertrophied infected nucleus (in) with virions (mc- marginated chromatin; nu-nucleolus; t-tubular structure) x 10,000 (B), x 6,000 (C).

D Hypertrophied nucleus with viral particles. Arrows indicate paracrystalline arrangement of virions (mc- remnants of marginated chromatin) x 12,000.

E Enlarged view of virions in the infected retinular cell nuclei of the infected compound eye of *P. indicus* affected with WSD. x 40,000

F Enlarged view of the C.S. of virions in the infected retinular cell nuclei of affected compound eye of *P. indicus* (e-envelope; nc-nucleocapsid) x 1,00,000

FIGURE 45



3.4 DISCUSSION

Transmission electron microscopic studies were conducted for five different tissues, namely, cuticular epithelium, gill epithelium, foregut, heart and compound eyes of *Penaeus indicus* affected with WSD. The results obtained are discussed below.

A few seemingly unaffected cells and nuclei were visible in all the tissues examined along with heavily infected nuclei in the nearby areas. Eventhough, such cells had nucleoli, diffused chromatin and nuclear membrane and a clear cytoplasmic membrane, most of the cytoplasmic organelles lost their structural integrity. Papathanassiou (1985) described the presence of abundant mitochondria in the epithelial cells of gills in normal decapoda, brown shrimp (*Crangon crangon*). Other vital components of actively functioning cells such as the golgi complexes, rough endoplasmic reticulum and numerous free ribosomes, occurring either singly or as polyribosomes were also present in these cells. The authors also observed a number of vesicles and microtubules, which occurred singly, or in bundles and traverse most of the cells. The epithelial cells in the present study did not exhibit such structures. Probably these organelles might have undergone degenerative process due to the growth of virions.

Hypertrophy of the affected nuclei and margination of the chromatin in them, were a common feature of WSBV infection in all the affected cells examined. According to Durand *et al.* (1996, 1997), these characteristics of the affected nuclei were indications of early WSBV infection and nuclear hypertrophy of the infected cells was due to the development and accumulation of intranuclear viral particles. Affected nuclei sometimes lied adjacent to each other

and clusters of hypertrophied nuclei were visible in some areas. In heavily infected nuclei, the nucleoli and nuclear membrane wereabsent.Similar reports were made by Wongteerasupaya *et al.* (1995a) and Durand *et al.* (1997) in *Penaeus monodon* infected with WSSV. Due to the absence of nuclear membrane, the contents of the affected nuclei were sometimes seen, simply dispersed in the cell, as an electron dense mass. The finely fibrillar and granular material, observed in the nucleoplasm seemed to correspond to the virogenic stroma. This was located in the central region of the nucleus. This might be probably formed by the disintegration of the nucleoplasm and chromatin, which were greatly reduced in quantity in the infected nuclei, compared to those in the healthy ones. Durand *et al.* (1997) observed linear and circular membranous structures, which they suspected to form *de novo* and formed the precursors of viral particles. Such circular membranous and linear structures were seen in the present study also. These structures did not have any contact with nuclear membranes. Hence it was probable that these structures were of viral origin.

The cytoplasmic organelles, such as endoplasmic reticulum, mitochondria underwent structural alterations. Swollen mitochondria, noted in the present case, lost their structural integrity. Similar observations were made by Papathanassiou (1985) in the epithelial cells of the gills in the brown shrimp, in a cadmium toxicity experiment. The functional cristae and their two bounding membranes were not clear in both the cases. In advanced stages, the hypertrophied mitochondria lost its structure, leaving vacuoles in their place. Similar observation was made by Kawase and Kurstak (1991) in the cells infected by Densonucleosis viruses (DNV), belonging to the family Parvoviridae. Papathanassiou (1985) suggested that the disruption of mitochondria membranes reduced the ability of mitochondria to synthesize ATP and led to an increase in

permeability, which might account for their swollen appearance. The metabolic activity of mitochondria was hampered severely after preparing the raw materials on a large scale for the multiplication of virions and their assembly within the nucleus. There could be energy dissipation within the host cells, due to the structural disruption of the organelles such as mitochondria, which are known as the power house of the cell. In the early stages of infection, rough endoplasmic reticulum were observed, indicating protein synthesis in the cell. Dilatation of endoplasmic reticulum, observed in the foregut epithelial cells in the present study was a characteristic feature of viral infection. Due to the disintegration of endoplasmic reticulum and ribosomal particles in heavily affected cells, there could be inhibition of host protein synthesis. The fine granular materials observed in the cytoplasm of heavily infected cells could be disintegrated fragments of the cytoplasmic organelles and microtubules. There could be alterations in almost all functions of the cell, such as enzymatic and ATP-ase activities, absorption and transportation of salts, active ion uptake, and protein synthesis and transport functions. Cytoplasmic disintegration and lysis led to large voids at lysed cell sites, in all the five tissues of *Penaeus indicus* examined. Similar observations were made by Wongteerasupaya *et al.* (1995a) in the epithelial cells of gills of *P. monodon* affected with SEMBV. Rajan *et al.* (2000) also reported that the number of mitochondria, endoplasmic reticulum and golgi bodies were reduced in white spot syndrome virus affected cells of penaeid prawns.

Rod shaped to elliptical, enveloped virions, typical of the non occluded, baculoviruses were observed in the hypertrophied nuclei of the affected cells in the subcuticular epithelium, gill epithelium, foregut, heart and compound eye tissues of *Penaeus indicus* infected with WSBV in the present study. Similar

observations have been reported by Wongteerasupaya *et al.* (1995a), Durand *et al.* (1996, 1997), Inouye *et al.* (1996), Momoyama *et al.* (1997), Wang *et al.* (1997a, 1997b), Kasornchandra *et al.* (1998) and Rajendran *et al.* (1999) in the gill and stomach epithelium of *P. monodon, P. japonicus, P. vannamei, Metapenaeus ensis* and crabs affected by WSBV. In the present study, two types of viral particles were observed. Virions having electron dense, cylindrical nucleocapsid and membranous envelope were seen. Other virions appeared faint and nucleocapsids were less electron dense. Those virions with highly electron dense, cylindrical nucleocapsid core and envelope, observed in the present study might be fully matured, complete virions, whereas, those with a faint or less electron dense core might be immature virions. According to Inouye *et al.* (1996) and Momoyama *et al.* (1997) complete virions were enveloped, ovoid to rod shaped and contained a partially lenticular shaped or cylindrical nucleocapsid with a highly electron dense core. Durand *et al.* (1997) opined that some enveloped caspids had a fine, electron dense, central, tenuous band, which appears like a filament. Present observations were in agreement with the findings of above cited authors.

Empty capsids, partially enveloped capsids and free nucleocapsids, without an envelope were also observed in the gill epithelium, sub cuticular epithelium, foregut epithelium and heart tissues of *Penaeus indicus*, infected with WSBV in the present case. Wongteerasupaya *et al.* (1995a) and Durand *et al.* (1996, 1997) made similar observations of nucleocapsids in different stages of development. The empty capsids were located in the central portion of the affected nuclei. These were usually enveloped, sometimes with an open extremity. Durand *et al.* (1997) also opined that in such cases, the space between the envelope and the capsid was reduced and the open end was large. In thin

sections, empty capsids were usually completely or partially enveloped and seldom completely naked. This was also true in the haemocyte infecting crustacean viruses (Johnson, 1988). Therefore, the morphogenesis of WSSV suggested that it was related to the haemocyte infecting crustacean viruses, described previously. However, the nucleocapsid development of the haemocyte infecting crustacean viruses, always occurred in association with membranes (Johnson, 1988). In contrast, the WSSV capsid was enveloped only after its formation. According to Durand *et al.* (1997) also, membranes enveloped empty capsids after its formation and before densification of the nucleocapsid core inside.

The sizes of the enveloped, mature virions (240-326 nm x 80-120 nm), cylindrical nucleocapsids (178-254 nm x 67-82 nm) and empty capsids (150-250 x 75-117 nm) observed in the present case in WSBV affected cells of *Penaeus indicus* were quite reasonable. Kasornchandra *et al.* (1998) and Rajendran *et al.* (1999) reported a size range of 240-340 nm x 70-120 nm and 210-320 x 80-100 nm respectively for enveloped virions and 182-250 x 60-80 nm for nucleocapsids of WSBV in penaeid prawns, which was similar to the observation made in the present study. Jasmin and Mary (2001) reported similar size range of WSBV in the retinular cell nuclei of compound eyes in *P. indicus*. The diameter of the empty capsid was less than that of the true nucleocapsid (with its component DNA material) as reported by Durand *et al.* (1997). White spot virions, detected by Wang *et al.* (1997a) in *Metapenaeus ensis* were smaller than those found in *P. monodon* and *P. japonicus*. Supamattaya *et al.* (1998) detected WSSV viral particles of similar size and shape in experimentally infected krill and crabs. Wongteerasupaya *et al.* (1995a) opined that the negatively stained virions were shorter and flatter than those, seen in thin sections. Huang *et al.*

(1994), Inouye *et al.* (1994), Nakano *et al.* (1994), Takahashi *et al.* (1994) and Chou *et al.* (1995) stated that negatively stained virions and nucleocapsids appeared to be larger than the reported measurements of the WSS viruses, that were obtained from the ultrathin sections of infected tissues. According to Takahashi *et al.* (1998), the sizes of the virions found in ultra thin sections were different among the viruses, such as PRDV, WSBV, SEMBV and HHNBV. This size difference could be attributed to different methods of preparing the samples and different measurement techniques being used in negatively stained preparations or in ultrathin sections. Sizes of the complete virions and nucleocapsids in the present study were in the same range as reported for other baculoviruses infecting crustaceans (Francki *et al.*, 1991).

Long tubules, measuring 530-720 nm x 80-118 nm size, were observed in the affected nuclei of the subcuticular epithelial cells in *Penaeus indicus*, affected with WSBV. Such observations were made by Durand *et al.* (1997) in the stomach and foregut epithelium of penaeid prawns, such as *P. monodon*, *P. vannamei* and *P. stylirostris*. They called these tubules as nucleocapsid precursors and noted 2 or 3 tubules placed side by side. In L.S., they exhibited segmentation that corresponded to the regularly spaced units measuring 16 nm in width. The diameter of the tubules was relatively closer to the diameter of the empty capsids and the segmentation of the tubules seemed to correspond to that of the nucleocapsid.

In highly infected nuclei, forming virions were located centrally, while mature virions tended to be concentrated along the nuclear margin in ordered arrays, displaying a paracrystalline arrangement in almost all the tissues examined in the present study. A few mature virions were seen randomly

scattered also. In some affected nuclei, these virions were arranged in small patches. Such an arrangement of matured virions was reported by Durand *et al.* (1997), Kasornchandra *et al.* (1998) and Rajendran *et al.* (1999) in WSBV affected nuclei of *Penaeus monodon* and *P. vannamei*. According to Durand *et al.* (1997), the assembled long tubules, which were assumed to be nucleocapsid precursors, broke into shorter tubules to form empty naked capsids. This pattern of formation could explain why mature virions blended into ordered arrays. In the WSBV affected retinular cell nuclei of compound eyes in *Penaeus indicus*, Jasmin and Mary (2001) reported such a paracrystalline arrangement of fully mature, enveloped virions with highly electron dense nucleocapsid core in them.

Superficial segmentation of the nucleocapsid observed in the negatively stained preparations of purified virions, reported by Wongteerasupaya *et al.* (1995a), Durand *et al.* (1996, 1997) and Sahul Hameed *et al.* (1998) was not observed in the present case. The authors reported a pattern of electron opaque bands, alternating with electron transparent bands, arranged perpendicular to the long axis of the extended nucleocapsid. In the present investigation, a few virions in the affected nuclei of subcuticular epithelial cells exhibited an envelope extension at one extremity. Durand *et al.* (1996, 1997) made similar observation in *Penaeus monodon* and this envelope extension or appendage was more prominent in negatively stained materials. According to the authors, the filamentous nucleoprotein entered the nucleocapsid through the open end of the capsid. After completing this process, the envelope extended as a sort of tail at this extremity and this was the ultimate step before completion of the mature virion. In the present study, even the nucleic acid core itself slightly projected into this envelope extension and this might be the extreme end of the filamentous nucleoprotein. A tail like structure was observed in all the virus strains (WSBV,

SEMBV and HHNBV), except PRDV (Inouye *et al.*, 1996). However, Takahashi *et al.* (1998) observed a tail like structure in negatively stained, purified PRDV virions by sucrose continuous density gradient. Lo and Kou (1998) also observed a tail like projection, extending from one end in some WSBV virions.

Structural asymmetry of the extremities of the nucleocapsids, as observed in the present study was reported by Durand *et al.* (1997) in *Penaeus monodon* affected with WSBV. This structural variation is a characteristic of the family Baculoviridae and has been observed in the non-occluded baculoviruses such as, Baculo- B and RV-CM (Johnson, 1988). The space between the envelope and the nucleocapsid exhibited variation in different development stages of the virions in the present study. In some virions, this area was very wide, whereas, in others it was narrow. Durand *et al.* (1997) also reported similar variation in the area between the envelope and the nucleic acid core in different virions. They suggested that the newly developed envelope was well separated from the capsid, but later became closely applied, except for a large open extremity. The bulging of the envelope in fully developed virions on both the parallel sides was to accommodate the laterally expanded nucleocapsid inside. The augmentation of the diameter between the empty capsid and the fully mature nucleocapsid could be due to a slight distention of the nucleocapsid near its centre, due to the maximal filling of the core by the filamentous nucleoprotein. The ultimate step before completion of the mature virions was the narrowing and the extension of the envelope as a sort of tail.

A characteristic feature of the mature virions was the presence of a trilaminar envelope around the highly electron dense nucleocapsid. The envelope observed in the present case consisted of two electron opaque layers, separated by

an electron transparent layer, which is a regular characteristic of baculoviruses. Similar observations of different layers of the envelope was reported by Wongteerasupaya *et al.* (1995a), Durand *et al.* (1997) and Wang *et al.* (1997b) in the white spot baculovirus observed in penaeid prawns, such as *P. monodon* and *Metapenaeus ensis*. The area observed between the nucleocapsid and the envelope in the present study (4-7 nm) agreed with the range reported for WSBV in the affected *P. monodon* by Durand *et al.* (1997). The thickness of the envelope of WSBV (5-7 nm), measured in the present study also perfectly agreed with the measurements made by Durand *et al.* (1997) in the WSBV from the stomach epithelial cells of *P. monodon*. In the C.S. of virions, the envelope of the mature virions were almost round or slightly oval in the present study, whereas, Wongteerasupaya *et al.* (1995a) reported even pentagonal or hexagonal shaped envelopes in the WSBV from *P. monodon*. Further studies are required, regarding the shape of the envelope. This might be probably due to the difference in strain or different developmental stages. Maeda *et al.* (1998a) experimentally confirmed the existence of an envelope in PRDV, by treating them with ethyl ether. The treated PRDV lost its infectivity. From this, they suggested that a virus, having an envelope lost its pathogenecity when treated with ethyl ether, because of the fat soluble envelope. Moreover, the presence of WSBV in the present study was confirmed only in tissues of ectodermal (epithelium of stomach, cuticle and gill) and mesodermal (heart) origin. The first step in a viral infection is the attachment of the virus to the outer surface of the host cell, and is performed by the membranes of enveloped viruses. The mechanism of the host cell membrane being recognized by the envelope of each virus is unique. This specificity is conferred by the binding properties of the membrane glycoproteins. Thus, the tissue specific tropism of particular viruses during infection is determined in many cases by the presence of a specific 'receptor' on the surface of

the susceptible cells, which is recognized by the relevant viral membrane protein. According to Wiley and Skehal (1990), receptor recognition provides a basis for viral tropism of both the enveloped and non-enveloped viruses.

Presence of WSBV virions in the cytoplasmic vacuoles of haemocytes, traversing the foregut epithelial layers was probably due to phagocytosis of the virions by the haemocytes. In the histopathological observations also, melanisation of the affected, necrotic areas were noted, which was part of the crustacean defense mechanism. Durand *et al.* (1997) also made similar observations and opined that though, the virions were seemed to be associated with membranous structures in the cytoplasm of the infected haemocytes, the hypothesis of intracytoplasmic multiplication of the virus seemed to be excluded, because the WSSV replicates in the nucleus. Presence of immature viruses with empty capsids, capsid originators, nucleocapsids, circular envelopes and membrane fragments in the nucleoplasm of the hypertrophied nuclei of the affected epithelial and heart cells of *Penaeus indicus*, indicated that multiplication and complete assembly of WSBV occurred within the nucleus. Similar observations made by Wongteerasupaya *et al.* (1995a), Durand *et al.* (1997) and Wang *et al.* (1997b) support this view.

Similarities that existed between WSBV and Nuclear Polyhedrosis Virus (NPV) were nuclear hypertrophy, disappearance of nucleoli and condensed chromatin, formation of virogenic stroma which appeared as a stripped area that became more condensed as infection progressed and lysis of infected cells. As the nuclear membrane disintegrated in advanced stages of infection, there was no clear distinction between the nuclear and cytoplasmic components in both the cases. Because WSSV morphogenesis was exclusively intranuclear, it was closer

to that of the nuclear polyhedrosis viruses, than that of the granulosis viruses. As in nuclear polyhedrosis viruses, the envelope was formed *de novo* in the nucleoplasm and development and replication of the virions were intanuclear. However, WSSV morphogenesis presented some peculiar characteristics: the WSSV capsid originated from the long tubules, which acted as naked empty nucleocapsid precursors or capsid originators. Although, the structure of the nucleocapsid and method of capsid development were unlike those described for other non occluded baculoviruses, the WSSV shared many characteristics with these viruses, including the nuclear localization, ultrastructure (shape, size, orientation etc.), morphogenesis and absence of a polyhedrin occlusion.

Another group of invertebrate viruses, to which WSSV bears resemblance, is the Densonucleosis viruses (DNV). Presence of virogenic stroma and paracrystalline array (linear array) of virions in the hypertrophied nuclei of the virus-infected cells was common to both. A similar crystalline array of virus particles was observed in the cytoplasm of the fat body, muscle and pericardial cells of *Periplaneta fuliginosa* infected with *Periplaneta* DNV (Suto , 1979). In the case of infection by Densonucleosis Viruses (DNV) also, chromatin was greatly reduced in amount and confined to the vicinity of the nuclear envelope as reported by Chao *et al.* (1985). But the viral particles in the both the cases differed very much in size. Densonucleosis viral particles were very small, measuring about 18-20 nm in diameter. Moreover, they belong to the family, Parvoviridae.

In almost all the tissues studied in *Penaeus indicus*, affected with white spot disease in the present case, nuclear hypertrophy, absence of nucleolus and nuclear membrane, margination of the disintegrated chromatin and presence

of virogenic stroma were the salient features of WSBV infection. Viral particles in various stages of development were observed in the virogenic stroma. Rod shaped enveloped virions measured 240-326 nm x 80-120 nm. Certain tubular structures measuring 530-720 nm x 80-118 nm were also observed. Immature viral particles were found in the central region of the affected nucleus and fully grown virions were arranged along the periphery of the affected nucleus, forming paracrystalline arrays. Cytoplasmic disintegration was also observed.

SUMMARY

SUMMARY

The various aspects of white spot disease (WSD) in penaeid prawns were studied in the present investigation. Five culture ponds of penaeid prawns were selected at Cochin. Ecological parameters were studied in all the ponds. Various symptoms of WSD were recorded in *Penaeus indicus*, *P. monodon* and *Metapenaeus dobsoni*. Histological observations were made and recorded in cuticular epithelium, gills, alimentary canal, heart and compound eyes of the normal and affected penaeid prawns. Ultrastructural studies were also carried out in the various tissues of *P. indicus* affected with WSD.

Water quality parameters such as temperature, salinity, dissolved oxygen, water pH, ammonia, nitrite, nitrate and phosphate in all the culture ponds were within the tolerance limits of prawns during the period of study. Soil quality parameters, such as soil pH and organic carbon were optimum in all the five ponds.

White spot disease (WSD) was observed in *Penaeus indicus*, *P. monodon* and *Metapenaeus dobsoni* of ponds P_1, P_2, C_1 and C_2. The occurrence of disease was frequent in ponds P_1 and P_2, which had an earlier history of WSD. The symptoms of WSD observed in the present study included reduction in food consumption, lethargy, sluggish erratic movements, pinkish to reddish discolouration of the body, gathering of the affected prawns along the pond margin, presence of white spots of 0.3 to 3.0 mm size on the inner surface of the exoskeleton and mass mortality within three to five days. A part of one or both the antennae of the infected prawns was lost. The exoskeleton in the affected prawns seemed to be rather loosely attached to the underlying epidermis, when compared to the healthy prawns.

The study conducted in the culture ponds showed that specimens of *Penaeus indicus* more vulnerable to WSD had 80 to 120 mm total length and 3.0 to 10 g body weight for females and 70 to 110 mm total length and 1 to 9 g weight for males. In the case of *P. monodon*, females having a total length of 75 to 115 mm and weight of 2 to 10 g and males having a total length of 75 to 110 mm and weight of 2 to 9 g were found to be more prone to WSD in culture ponds. Size groups of *Metapenaeus dobsoni*, easily affected by WSD had 60 to 80 mm total length and 1.5 to 4g weight for females and 60 to 80 mm total length and 1.5 to 3 g weight in the case of males. In the experimental study, mass mortality of *P. indicus* occurred on the fifth day of feeding them with infected prawn meat. Whereas, in the case of *P. indicus*, reared in water and soil taken from affected ponds and fed with healthy prawn meat, mass mortality occurred on the seventeenth day of stocking.

Tissues affected with white spot disease were ectodermal and mesodermal in origin. Midgut and hepatopancreatocytes were unaffected by WSD, as they were of endodermal in origin. Marked histopathological changes were observed in the subcuticular epithelium, gills, hindgut, antennae and pleopods and heart. Haemocytes and connective tissues were also found affected by WSD. Retinular cell nuclei region of compound eyes of penaeid prawns was found to be one of the main target organs of WSD. Necrosis, cellular lesions and structural disintegration were severe that it might invariably led to functional disorders. Presence of eosinophilic and basophilic inclusion bodies in the markedly hypertrophied nuclei of affected cells was the foremost histopathological change observed in the present study. Eosinophilic and basophilic inclusion bodies were indications of initial and final stages of infection respectively. Nucleoli and nuclear membranes were absent in the affected nuclei. In severe cases of infection, cytoplasmic membranes were disintegrated and

several hypertrophied nuclei with intranuclear inclusions were found to cluster together. The sizes of the hypertrophied nuclei ranged from 3 to 14 μm in diameter, as against 2.6 to 7 μm in healthy condition.

Ultrastructural studies were conducted in the subcuticular epithelial cells of exoskeleton, gills, stomach, myocardial cells and retinular cell nuclei region of the compound eyes of *Penaeus indicus* affected with white spot syndrome virus (WSSV). All the infected nuclei were highly hypertrophied. The nucleoli and nuclear membranes were completely lost. The chromatin became marginated and disintegrated, resulting in the formation of virogenic stroma in the affected nuclei. In the virogenic stroma, viral development and assembly occurred, resulting in the formation of fully-grown virions. Tubular and circular structures, empty capsids, nucleocapsids and partially formed envelopes observed in the affected nuclei were indications of viral development in the virogenic stroma. White spot syndrome virions were rod shaped, enveloped and with a highly electron dense nucleocapsid inside. The envelope was trilaminar, having two electron dense layers, separated by an electron lucent layer in between. The size of the virions ranged from 240 to 326 nm in length and 80 to 120 nm in width. The tubular structures measured 530 to 720 nm in length and 80 to 118 nm in width. Mature viral particles were released into the surrounding cytoplasm for fresh infection. Large voids were observed in the affected cell, indicating disintegration of the cellular organelles. Cytoplasmic organelles such as rough endoplasmic reticulum, ribosomes, mitochondria, golgi complex etc. were found disintegrated. Immature viral particles along with the virogenic stroma were found in the centre of the infected nuclei. Mature virions arranged themselves along the periphery of the infected nuclei, resulting in the formation of paracrystalline arrays.

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